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State-of-the-Art Review

Experimental Culture Conditions Are Critical for Ex Vivo Expansion of Hematopoietic Cells

LUC DOUAY

ABSTRACT

The ex vivo expansion of hematopoietic stem cells (HSC) for clinical use is now recognized to be a feasible and very promising approach for hematotherapy. Expansion of specific HSC subsets is required for different clinical applications, for example, to increase the number of mature cells, to produce specific cells for adoptive therapy, or to increase the number of primitive stem cells available for engraftment. Although hematopoietic growth factors can play an important role in this setting, in this review we emphasize that other variables affect the outcome of stem and progenitor cell expansion. These variables include the serum supplement, the purity of $CD34^+$ cells, the initial cell concentration, and the duration of culture. It is also essential to define standard culture conditions for normal stem cells and to limit or prevent expansion of residual tumor cells. In clinical applications, determination of the hematopoietic value of the expanded population is mandatory. Thus, we have to demonstrate the expansion of primitive hematopoietic progenitor and stem cells, with maintenance of their hematopoietic potential as assessed by in vitro or in vivo assays. We draw attention to the challenges in the clinical application of ex vivo expansion. These include the establishment of well-defined experimental conditions and the determination of the hematopoietic value of the expanded grafts, whatever the graft source: bone marrow, mobilized peripheral blood, or cord blood. Future studies hopefully will optimize these procedures and allow not only expansion but engineering of defined cellular functions as HSCs grow under defined conditions.

INTRODUCTION

THE CLINICAL INTERESTS of the ex vivo expansion of hematopoietic stem cells (HSCs) are numerous. Our aim may be to increase the number of mature cells, to produce specific cells for adoptive therapy, or to increase the number of primitive stem cells. Thus, the expansion of specific hematopoietic cell subsets is required for different clinical applications in the domains of HSC transplantation, immunotherapy, and productive gene therapy. A major issue in HSC expansion is the question of whether the different combinations of hematopoietic growth factors (HGFs) employed provide amplification of the progenitor, precursor, or primitive HSC pool. The culture conditions and HGF mixture indeed largely determine the final composition of in vitro-expanded cells (1). Ex vivo expansion has been reported to be stromal cell-dependent (2), but stroma-free culture systems with recombinant human HGFs are still most commonly used for ex vivo expansion of HSCs (3).

Although HGFs play an important role in the number and type of cells generated in stroma-free static ex vivo cultures, other variables may also affect the outcome of stem and progenitor cell expansion. These include the autologous serum supplement or serum-free substitutes, the purity of $CD34^+$ cells, the initial cell concentration, and the duration of culture (1,4). The challenges in the clin-

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ical application of ex vivo expansion are the establishment of well-defined experimental conditions and the determination of the hematopoietic value of the expanded grafts, whatever the source of HSCs, bone marrow (BM), mobilized peripheral blood (PB), or cord blood (CB).

A DEFINED CULTURE MEDIUM HAS TO BE ESTABLISHED

In experimental procedures, media containing xenogenic supplements like fetal calf serum (FCS) or bovine serum albumin (BSA) are most widely employed, although use of these additives may not be permitted in clinical applications for infectious reasons. Moreover, all biological reagents must be tested for their innocuity and the absence of infectious agents. The culture media for clinical applications need to be completely defined and standardized. Because commercially prepared complete media without animal proteins could help to provide more reproducible results, several teams have investigated the possibility of using culture media free of animal proteins (5). In our laboratory, the replacement of animal serum [FCS + horse serum (HS)] by human plasma was not detrimental to overall results. This is in slight contrast to frequent reports in the literature that low concentrations of autologous plasma (1–2%) are sufficient to support HSC expansion (6,7). Our experiments indicated that a higher concentration of human plasma (10%) was required to provide an adequate substitute for FCS + HS. One possible explanation is that in most of the investigations published to date, the plasma was a by-product of leukapheresis and the cytokines interleukin-6 (IL-6) and granulocyte colony-stimulating factor (G-CSF) produced by monocytes and T cells during hematopoietic recovery following intensive chemotherapy might interact with the cytokines added in vitro. In an attempt to eliminate irrelevant cytokines or inhibitors, we studied the expansion of normal $CD34^+$ cells in the presence of a previously described serum-free substitute (8,9) and found that this substitute could be preferable to animal serum or human plasma. Thus, total cell and $CD34^+$ cell expansion increased more than three-fold in serum-free media as compared to media containing 1–10% human plasma or 25% animal serum, whereas colony-forming units granulocyte-macrophage (CFU-GM) expanded four-fold and burst-forming units erythroid (BFU-E) three-fold and long-term culture–initiating cells (LTC-IC) were maintained. Similar results were obtained for human plasma concentrations of up to 25%, although this is not clinically relevant because a 400-ml blood sample would be required in an autologous situation. A serum-free substitute is therefore a good candidate for the supplementation of long-term culture media, provided BSA can be replaced by human serum albumin in clinical applications (1).

STEM CELL SELECTION AND STEM CELL LOSS

The expansion capacity of a given HSC sample would be best exploited by starting with a population of cells selected on the basis of their known immunophenotypic distribution. However, under present experimental conditions, the isolation of subpopulations with rare phenotypes results in the loss of a variable proportion of these cells. One needs to strike a balance between the expansion potential of the selected subsets and the number of cells lost during purification. A specific cell population may be required for ex vivo expansion, whereas depending on the goals both enrichment of the desired population and purging of an unwanted cell fraction are currently possible.

There are few comparative data on the expansion capacity of hematopoietic progenitors in samples of mononuclear and selected cells. Sandstrom et al. (10) found that perfusion strongly increased progenitor expansion and LTC-IC maintenance, whereas $CD34^+$ selection had in itself no beneficial effect. The best results were that PB mononuclear cells expanded 50-fold in total cell number, 80-fold in CFU-GM, and 20-fold in LTC-IC in a bioreactor in the presence of four HGFs (11).

Initial studies of the expansion of HSCs showed that cell proliferation was highest using $CD34^+$ cells as the starting population, which suggests that the presence of more mature cells has a strong suppressive effect on the expansion of progenitors, particularly in static cultures where all cellular by-products remain in culture. Several groups have shown that the presence of inhibitory accessory cells in BM or PB limits the degree of expansion of the progenitor compartment in ex vivo cultures. In addition, the selection of $CD34^+$ HSCs is of major importance not only to obtain an enhanced proliferative capacity but also for considerations of volume, purging, and use in gene therapy. Although they are widely proposed, it is not clear that protocols for enrichment of immature cells are mandatory prior to culture. Using immunomagnetic or immunoadsorption techniques, there is an average loss of 2–3 logs for total cells and of 50% for progenitor/LTC-ICs (12). Consequently, the ex vivo expansion of $CD34^+$ cells must be at least 500-fold for total cells and 10-fold for progenitor/stem cells to be of clinical relevance.

INITIAL CELL CONCENTRATION AND FINAL EXPANSION

Because in static cultures the initial cell concentration considerably influences the number of cells it is possible to generate, a laboratory should determine the kinetics of cell growth in its cultures and use adequate initial con-

centrations to obtain optimal numbers of viable cells. Haylock et al. (13) observed a consistent exponential growth rate, irrespective of their initial concentration of $CD34^+$ cells. However, under their culture conditions, cell death increased and the total number of viable cells decreased when the cell density was above $1.1–1.4 \times 10^6$/ml. Hence, it is important that each laboratory determine the initial cell concentrations that generate optimal numbers of the desired subpopulations from a given starting cell population under different culture conditions. Using bone marrow cells, we showed that the initial concentration can affect the expansion of all hematopoietic compartments. Thus, its reduction from 3×10^4 to 1.5×10^3/ml increased the total cell expansion almost 20-fold and progenitor expansion more than three-fold (1). In the case of primitive cells, our data confirm the observations of Shapiro et al. (14) concerning late precursors in cultures of 4 or 2×10^4 cells/ml. These results are important for clinical applications. In short, cell proliferation and the production of up to several hundred times the initial number of cells lead to the depletion of metabolic substrates in the medium, a modification of the pH, and the accumulation of waste products and inhibitors. The medium depletion is aggravated by reduction of cytokines and oxygen, and, when cytokines are added, these problems of nutrient depletion are further exacerbated by the several-fold enhancement of cell proliferation (15).

CULTURE DURATION: A TECHNICAL LIMITATION

Haylock et al. (13) were the first to succeed in expanding hematopoietic stem cells and progenitors from highly purified $CD34^+$ cells isolated from mobilized PB. The cultures displayed two phase of growth. If pre-CFU and committed progenitor cells were predominant during the first 7–10 days, numbers of mature myeloid cells at all stages of differentiation increased and CFU-GM production simultaneously decreased from day 10 onwards. There was a mean 1324-fold expansion of MNCs over 21 days of culture, whereas CFU-GM production was highest on day 14 with a mean 66-fold amplification. In our laboratory, human BM cells expanded for 10 days remained capable of producing precursors, progenitors and LTC-ICs for more than 100 days, in up to 80,000-, 7,000-, and 80-fold quantities, respectively (16). This reflects at least the persistence and probably the expansion of primitive stem cells, which, in turn, suggests that these populations could undergo self-renewal divisions. Similarly, Piacibello et al. have reported that all hematopoietic lineages can be continuously and increasingly generated for 6 months, with levels of amplification of up to 2,000,000-fold for CFU-GM and 200,000-fold for LTC-IC (17). These continuously increasing levels of expansion were nevertheless obtained in preclinical models. In hematopoietic cell cultures established under clinically relevant experimental conditions, the limited productivity after 14 days is certainly due in part to excessively high cell concentrations in the starting cultures, aggravated by suboptimal feeding protocols. Many studies have indeed reported a decrease in progenitor production during days 12–15 of culture (4), indicating that expansion in static liquid cultures should be limited to 7–14 days. This problem of adapting cell culture conditions to accommodate the considerable cell proliferation might in the future be circumvented by the development of dynamic expansion systems.

PURGING OF MALIGNANT CELLS?

One of the theoretical interests of the ex vivo expansion of cells for clinical transplantation is the reduction of the volume of blood or bone marrow to be collected from the patient and, hence in cancer therapy, the reduction of the number of tumor cells in the final graft samples (18). The kinetics of tumor cell growth vary according to the type of tumor and for a given type from one patient to another. In cancer patients, ex vivo-expanded hematopoietic cells should therefore be monitored for minimal residual disease. Moreover, ex vivo HSC expansion for autologous use can in this situation only be advantageous if contaminating tumor cells are not expanded concomitantly. PB cells seem to fulfil this requirement. In a study of PB cells before and after ex vivo expansion in breast cancer patients, the number of pathological cells increased 45-fold whereas that of normal $CD34^+$ cells increased 110-fold after 14 days co-culture (19). However, when the cultures were maintained for longer periods of time, tumor cells were able to regrow. Others found no tumor cell expansion in cultures of PB progenitor cells derived from patients with multiple myeloma (20) and concluded that grafts obtained by ex vivo expansion of $CD34^+$-selected PB cells from cancer patients do not contain detectable tumor cells. This would suggest there is no increased risk of concomitantly expanding pathological cells. Widmer et al. (21) observed that in ex vivo expansion cultures of seven BM and two PB samples collected from non-Hodgkin's lymphoma patients with t(14;18) translocation-positive tumor cells did not proliferate and became almost undetectable in most cases. In our laboratory, we investigated the ex vivo expansion capacity of $CD34^+/CD19^-$ cells in patients with bcl2/JH positive non-Hodgkin's lymphoma. After $CD34^+$ and $CD19^-$ selection, the majority of samples were positive for the bcl2/JH rearrangement. In all positive cases, $bcl2/JH^+$ cells were, however, no longer detectable after expansion, whereas total cells, CFU-GM, BFU-E, and LTC-IC were expanded by up to 3248-, 92-,

28-, and 6-fold, respectively (22). These findings point to the possible use of negative or positive tumor cell purging systems in association with ex vivo expansion to reduce the risk of contamination by pathological cells.

To date in our hands, taking into account all the abovementioned parameters, the most efficient culture conditions appear to be the following: $CD34^+$-selected cells, at an initial concentration of 5×10^4/ml for BM cells or 1×10^4/ml for CB cells, grown in a serum-free, stroma-free system in gas-permeable polypropylene bags for 14 days. The combinations of cytokines we found to give high-level expansion of all hematopoietic compartments were: stem cell factor (SCF) (100 ng/ml), Flt3-L (100 ng/ml), megakaryocyte growth and development factor (MGDF) (100 ng/ml), G-CSF (10 ng/ml), IL-6 (10 ng/ml), and IL-3 (5 ng/mL) for BM cells and SCF, Flt3-L, MGDF, and G-CSF for CB cells (1,16,23–25).

THE SHORT- AND LONG-TERM ENGRAFTMENT CAPACITY OF THE MANIPULATED GRAFT HAS TO BE EXPLORED

In clinical applications, determination of the hematopoietic value of the expanded population is mandatory. Its aim is to demonstrate the expansion of primitive hematopoietic progenitor/stem cells, with maintenance of their hematopoietic potential as assessed by in vitro assays and of the nonobese diabetic severe combined immunodeficiency (NOD-SCID) in vivo repopulating capacity.

An assessment of the functional capacity of expanded neutrophils and monocytes requires testing of their phagocytic activity and oxidative metabolism, two properties characterizing the terminal stages of functional granulocyte differentiation (23). The expanded cells produced in our cultures displayed lower but nonnegligible phagocytic activity as compared to normal control cells. A study of their potential to mediate oxygen-dependent microbe killing through production of hydrogen peroxide showed that the expanded cells generated hydrogen peroxide upon stimulation by phorbol myristate acetate (PMA), although in lesser quantities than control BM cells. Cultures initiated at 5×10^4 cells/ml produced slightly higher numbers of functional cells than those initiated at 10^5 cells/ml, but with a similar responsive population. The cultured cells were also capable of adhering to an FCS-coated surface after stimulation with PMA and of migrating toward chemotactic agents. Overall, these results suggest that ex vivo-expanded cells have the ability to perform the biological functions essential to host defense against bacterial infection, even if their efficiency is not equivalent to that of control cells from normal bone marrow. The differences in functional capacity between expanded and nonexpanded cells probably reflect various effects of in vitro cell differentiation on the terminal maturation process. Interferon-γ (IFN-γ) could be used to induce the terminal maturation of cultured cells, as it has been reported to stimulate most of the functional activities of neutrophils and monocytes.

The primitive functions of expanded cells can be tested in vitro in clonogenic cell (CFC [colony-forming cell], LTC-IC, and E-LTC-IC [extended-LTC-IC]) (26,27) and lymphopoiesis assays natural killer [(NK), B, and T] (28,29,31) and in vivo by evaluating long-term engraftment of the bone marrow of NOD-SCID mice (32–34). Using in vitro assays, we showed that under our experimental conditions the mean levels of expansion of BM cells were 200-fold for total cells, 70-fold for CFU-GM, and 30-fold for LTC-IC (16) and that lymphoid B and NK progenitors were present (25). Similarly, CB cells could be expanded by up to 1613-fold for total cells, 278-fold for CFU-GM, 47-fold for LTC-IC, and 21-fold for E-LTC-IC (24), with detectable lymphoid B, NK, and T progenitors (24). When such expanded CB populations were transplanted into NOD-SCID mice, they were able to generate myeloid progenitors and lymphoid cells for up to 5 months. These primitive progenitors engrafted the NOD-SCID bone marrow, which contained LTC-IC at the same frequency as that of control transplanted mice, with conservation of their clonogenic capacity. Moreover, human $CD34^+$ $CD19^-$ cells sorted from the engrafted marrow were able to generate $CD19^+$ B cells, $CD56^+$ $CD3^-$ NK cells and $CD4^+$ $CD8^+$ $\alpha\beta TCR^+$ T cells in specific cultures. Our expansion protocol also maintained the telomere length in $CD34^+$ cells, owing to a nine-fold increase in telomerase activity over the 2 weeks of culture (24). Such experiments provide strong evidence that expanded $CD34^+$ CB cells retain their ability both to support long-term hematopoiesis, as shown by their engraftment in the NOD-SCID model, and to undergo multilineage differentiation along all myeloid and the B-, NK-, and T-lymphoid pathways.

It is of major importance for the clinician to collect all parameters relating to the expanded product before its infusion into the patient. In our hands, it was possible to cryopreserve expanded progenitor/stem cells with 70–90% recovery, although as expected (23) the mature cells were less well recovered (45%). At the functional level, the cryopreserved cells also maintained their proliferative capacity. Hence the freezing step may be considered to be no more than a blocking stage, without any incidence on the ability of the cells to further expand in vivo. Nevertheless, as one cannot ignore the impact of the loss of mature cells after thawing, which will probably influence the short phase of hematopoietic recovery, evaluation of the expanded graft prior to its reinfusion remains of considerable interest to facilitate the planning of clinical and laboratory requirements.

CONCLUSION

The ex vivo expansion of hematopoietic cells for clinical use is now recognized to be a feasible and very promising approach (35–40). However, it is essential to define reproducible and reliable standard culture conditions because several parameters, not all of which can be controlled in static liquid systems, play a critical role in this setting (41). This will require new expansion systems such as bioreactors mimicking the in vivo situation, where it is possible to adjust the culture conditions to the level of cell proliferation. Combinations and doses of HGFs and their sequential use are other critical variables influencing the outcome of strategies for ex vivo expansion, as is the source of HSCs. One must also emphasize the necessity of establishing experimental conditions specific for the clinical goal, namely expansion of the primitive pool or of the precursor/progenitor compartment. Finally, we need to use reliable assays to evaluate the capacity of cultured human hematopoietic cells to ensure short- and long-term engraftment following transplantation.

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Stem cells and repair of lung injuries

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Abstract

Fueled by the promise of regenerative medicine, currently there is unprecedented interest in stem cells. Furthermore, there have been revolutionary, but somewhat controversial, advances in our understanding of stem cell biology. Stem cells likely play key roles in the repair of diverse lung injuries. However, due to very low rates of cellular proliferation *in vivo* in the normal steady state, cellular and architectural complexity of the respiratory tract, and the lack of an intensive research effort, lung stem cells remain poorly understood compared to those in other major organ systems. In the present review, we concisely explore the conceptual framework of stem cell biology and recent advances pertinent to the lungs. We illustrate lung diseases in which manipulation of stem cells may be physiologically significant and highlight the challenges facing stem cell-related therapy in the lung.

Introduction

According to Greek mythology, the immortal Prometheus stole fire from the Gods as a gift for humankind. As punishment, he was shackled to a rock, whereupon each day for 30,000 years an eagle consumed as much of his liver as would regenerate. There is some debate whether the eagle ate his liver or heart, but what if the bird had a taste for lung? And what if Prometheus was a mere mortal?

Analogous to Prometheus and the eagle, the ambient air-exposed lung is subject to an array of potentially damaging agents, including chemical oxidants and proteolytic enzymes. Presumably, daily oxidant and protease wear and tear on structural components such as elastin and collagen contributes to inevitable age-related declines in pulmonary function in normal individuals [1,2]. Acute and chronic lung disease, or its treatment with oxygen and positive pressure ventilation, may further damage lung tissue in excess of the capacity for orderly repair, resulting in characteristic pathologic changes including tissue destruction or fibrotic scarring [3-5]. But what determines the lungs' capacity for repair? Certainly, one factor must be the ability of stem cells to proliferate and differentiate to replace damaged cells and tissues. As discussed later in this review, the traditional view is that, during development, self-renewing tissues are imbued with resident, tissue-specific stem cells, so-called adult somatic stem cells. However, recent but highly controversial evidence suggests that stem cells from one type of tissue may generate cells typical of other organs. In this fashion, circulating

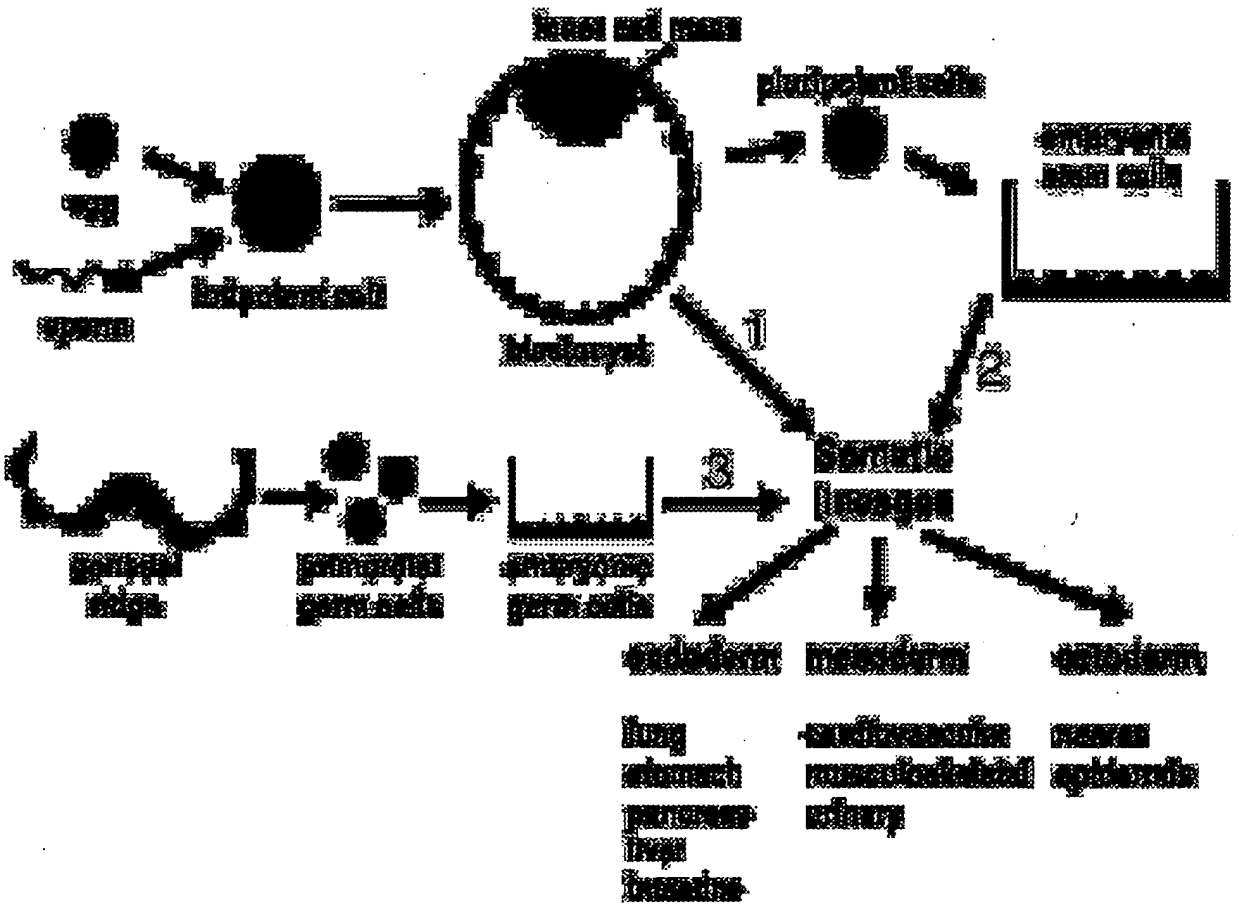

Figure 1
Cell lineage determination during embryogenesis and generation of pluripotent embryonic cells. The three primary germ layers form during normal development (path 1). Embryonic stem cells from the inner cell mass (path 2) or embryonic germ cells from the gonadal ridge (path 3) can be cultured and manipulated to generate cells of all three lineages.

cells derived from bone marrow may augment resident stem cells, and we comprehensively review such data from lung. Finally, there is great hope that embryonic stem cells, embryonic germ cells, or even adult somatic stem cells can be engineered as an unlimited source of cells to enhance organ-specific repair or replace lost tissues. Below, we concisely review stem cell biology, focusing on recent findings relevant to the lungs. Diseases in which alterations in stem cells contribute to lung dysfunction are discussed, as are the challenges facing the nascent field of pulmonary regenerative medicine.

Embryonic and adult (somatic) stem cells

For links to more in-depth information on general principles in stem cell biology, a comprehensive glossary, and the latest updates in this quick moving field, the reader is referred to the International Society for Stem Cell Biology http://www.isscr.org. During embryonic development, the inner cell mass of the blastocyst forms three primary germ layers, which generate all fetal tissue lineages (reviewed in [6], illustrated in Figure 1, path 1). Embryonic stem cells (derived from the blastocyst inner cell mass), or embryonic germ cells (derived from the gonadal ridge), when cultured on embryonic mouse fibroblast feeder cell layers in the presence of a differentiation-sup-

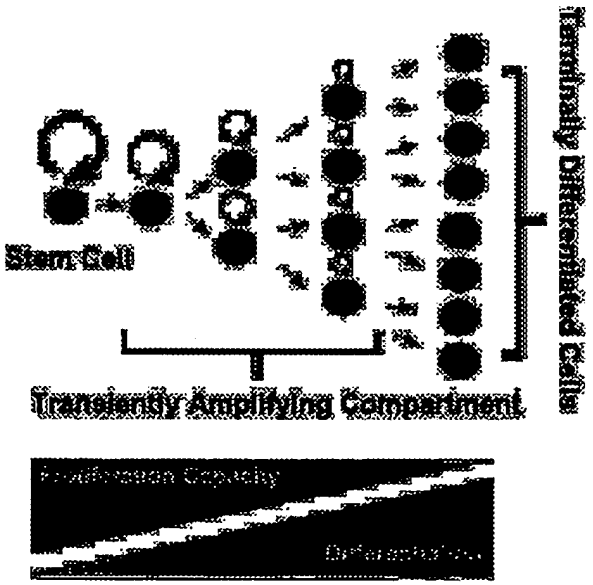

Figure 2
Traditional view of cell lineage in adult renewing tissues. Organ-specific (somatic) stem cells generate characteristic cell types through a linear set of commitment and differentiation steps. Arrow thickness represents self-renewal potential.

pressing cytokine (leukemia inhibitory factor), proliferate indefinitely and remain pluripotent. Manipulation of culture conditions can coax the cells to undergo differentiation characteristic of many tissue types (Figure 1, paths 2 and 3). Theoretically, pluripotent embryonic cells can serve as an unlimited resource for therapeutic applications [7,8].

General principles of tissue renewal by adult stem cells have been reviewed recently [9] and can be summarized as follows. The traditional view of cell lineages is that adult somatic stem cells maintain cell populations in adult tissues. The adult lung falls into the category in which cell proliferation is very low in the normal steady-state but can be induced dramatically by injury (see [10,11] for recent reviews of lung stem cells). The conditional nature of lung cell proliferation complicates the search for lung stem cells. Cell lineages are much better understood in continuously proliferating tissues such as the gut, skin and hematopoietic system (reviewed in [12-14], respectively). The long-standing view, developed from these other organs, is that stem cells reside in well-protected, innervated, and vascularized niches that provide cues regulating cell fate decisions such as proliferation, migration, and differentiation [15]. Adult stem cells are capable of abundant self-renewal and can also generate the specific cell lineages within the tissue compartment (Figure 2). Proportional to tissue needs, stem cells may undergo asymmetric cell division, in which they generate one stem cell and a committed progenitor. The capacity for self-renewal decreases progressively as committed progenitors differentiate. The wisdom of the body is to conserve stem cells. They cycle infrequently and the majority of cell replacement is accomplished by committed progenitors within the so-called transiently amplifying compartment. Eventually, individual cells become incapable of further cell division. In tissues, there are specific temporal and spatial hierarchic relationships between stem cells in their niches and their differentiated progeny. Within this axis, cell proliferation, migration, differentiation, function, death, and removal are tightly regulated to maintain tissue homeostasis.

Cell compartments in the lung and functional integration

In the architecturally complex lung, cells of multiple germinal lineages interact both during morphogenesis and to maintain adult lung structure. Even within derivatives of a single germ layer, cells become subdivided into separate cell lineage "zones". For example, the endoderm generates least four distinct epithelial regions, each with a different cellular composition (Figure 3). Additional cell types, including airway smooth muscle, fibroblasts, and the vasculature, are derived from mesoderm. Airway and alveolar architecture, and in turn, function, result from interaction among epithelium, smooth muscle, fibroblasts, and vascular cells, all within an elaborate structural matrix of connective tissue. The complexity of even this oversimplified view, which omits pulmonary neuroepithelial cells and bodies, innervation, and classical hematopoietically-derived cells such as dendritic cells, mast cells, and macrophages, has hindered identification of lung stem cells and patterns of cell migration during tissue renewal. Nevertheless, the prevailing view is that airway basal and Clara cells and alveolar type II cells serve as epithelial progenitors [11,16-19]. Cell lineages in the mesodermal compartments remain less well understood.

Stem cell plasticity and the lung

Recent studies challenge the view that tissues are maintained solely by organ-specific stem cells. There is evidence that adult stem cells from a variety of sources can generate not only their own lineages, but those of other tissues, sometimes crossing barriers of embryonic derivation previously thought impenetrable [20,21,8]. There are a few controversial reports that adult stem cells from outside the bone marrow may reconstitute the hematopoietic system, but most of the evidence flows in the other direction- namely, that cells from the bone marrow can generate diverse non-hematopoietic cell types. Both

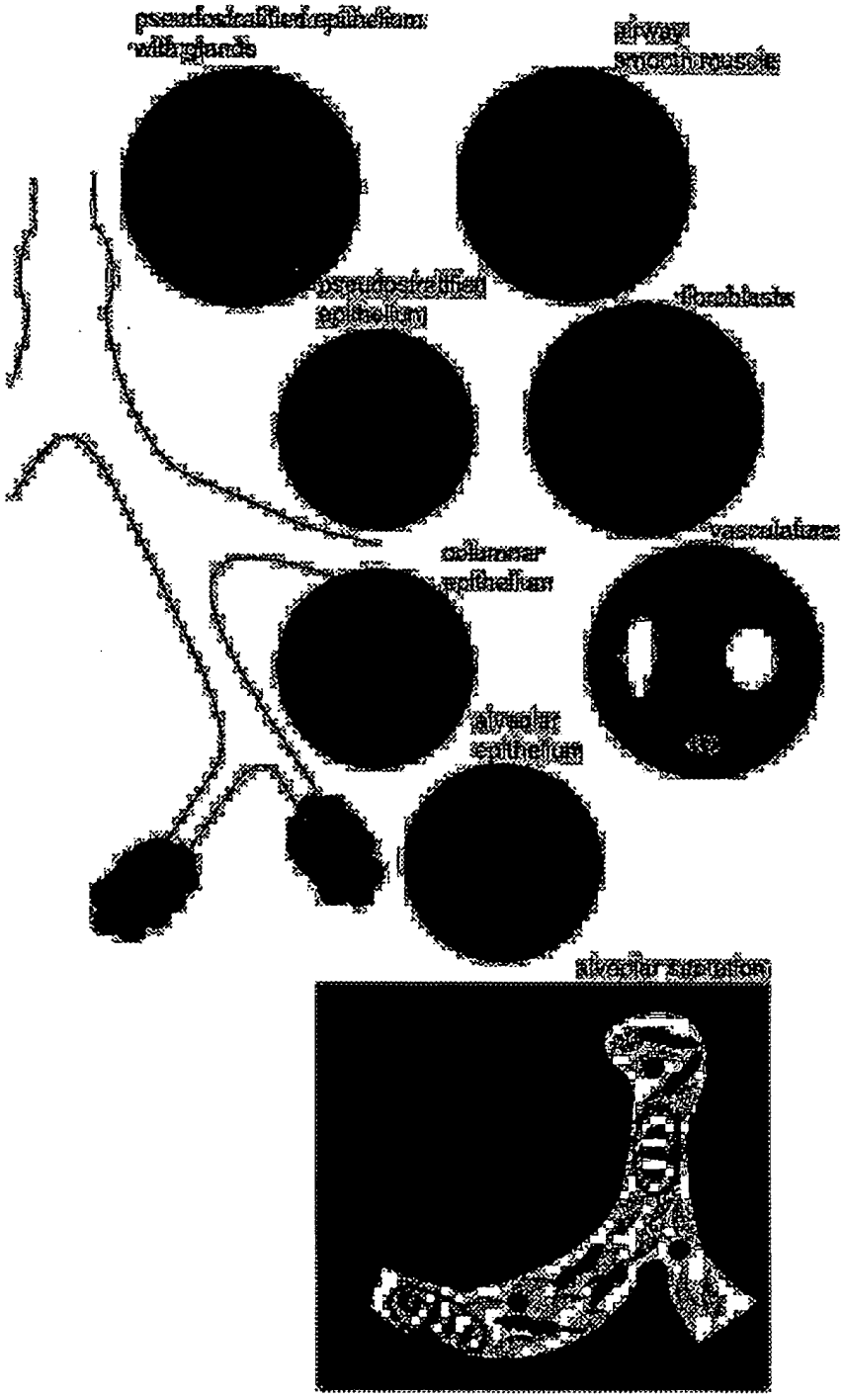

Figure 3
Stem cell compartments in the lungs. The endoderm-derived epithelium can be subdivided into at least 4 types whereas smooth muscle, fibroblasts, and vascular cells are derived from mesoderm. The coordinated interaction of multiple cell types, including alveolar epithelium, interstitial fibroblasts, myofibroblasts and pulmonary endothelium, is necessary to form alveolar septa.

experimental studies in animals and human clinical studies, summarized in Table 1, provide evidence for, and against, circulatory delivery of lung progenitor cells. While bone marrow-derived cells, such as alveolar macrophages, dendritic cells, mast cells, and lymphocytes, normally migrate to the lung, the surprise in the recent literature is that under certain circumstances circulating cells can apparently generate lung resident cells, including epithelial, endothelial, and myofibroblast cells. The technical approach towards identification of these cells is often technically challenging and involves co-localization of a donor cell marker, for example, the Y chromosome, in sex-mismatched transplantation, or a genetically engineered marker in mouse experiments, and proteins characteristic of the differentiated cell type in the lung, for example, keratin in epithelial cells or collagen in fibroblasts. As discussed below, the results are highly variable and often contradictory, depending on factors including the starting cell population, the methods for marker detection, and the amount of injury to the lung.

Table 1: Evidence for, and against, circulating progenitor cell generation of non-hematopoietic lung cell types.

Study Type	Disease or Model	Tissue of Origin	Lung Cell Type Formed / Frequency	Method of Detection	Ref.
Animal, *in-vivo*	BMT	MSC	Undefined mesenchymal cells / occasional	PCR for collagen gene marker	[30]
Animal, *in-vivo*	Bleomycin fibrosis	MSC	Type I pneumocytes / rare	β galactosidase protein	[23]
Animal, *in-vivo*	BMT	HSC enrichment	Type II pneumocytes / up to 20%, bronchial epithelium / 4%	Y chromosome FISH, surfactant B mRNA	[31]
Animal, *in-vivo*	Radiation pneumonitis	Whole bone marrow	Type II pneumocytes, bronchial epithelium / up to 20% of type II cells	Y chromosome FISH, surfactant B mRNA	[25]
Animal, *in-vivo*	BMT	Whole bone marrow/ EGFP retrovirus	Type II pneumocytes / 1–7%	EGFP, keratin immunostain, surfactant protein B FISH	[33]
Animal, *in-vivo*	BMT and parabiotic animals	HSC	Hematopoietic chimerism but exceedingly rare lung cell types	EGFP	[32]
Animal, *in-vivo*	Bleomycin fibrosis	MSC	Type II pneumocytes / ~1%	Y chromosome FISH	[22]
Animal, *in-vivo*	Radiation fibrosis	MSC or whole bone marrow	Fibroblasts / common	EGFP, Y chromosome FISH, vimentin immunostain	[26]
Animal, *in-vivo*	BMT	Bone marrow, EGFP labeled	Fibroblasts, Type I pneumocyte / occasional to rare	Flow cytometry	[34]
Animal, *in-vitro* and *in-vivo*	Hypoxia-induced pulmonary hypertension	Circulating BM-derived c-kit positive	c-kit positive cells in pulmonary artery vessel wall; In hypoxia, circulating cells generate endothelial and smooth muscle cells *in-vitro*	Flow cytometry and immunohistochemistry	[27]
Animal, in-vivo	Ablative radiation and elastase induced emphysema	GFP + fetal liver	Alveolar epithelium and endothelium; frequency not reported but increased by G-CSF and retinoic acid	Immunohistochemistry for $CD45^-$, GFP^+ cells	[28]
Animal, in-vivo	Bleomycin fibrosis	Whole marrow GFP^+	GFP^+ type I collagen expressing	Flow cytometry and immunohistochemistry, RT-PCR	[24]
Human, *in-vitro*	Heat shock in cell culture	MSC and SAEC	Cell fusion / common	Immunostaining, microarray	[39]
Animal, *in-vivo*	OVA-sensitized mouse model	CD34 positive, collagen I expressing fibrocytes	Myofibroblasts / ?	CD34-positive, collagen I, α-smooth muscle actin	[29]
Human, *in-vivo*	Allergen – sensitized asthmatics	CD34 positive, collagen I expressing fibrocytes	Myofibroblasts / ?	CD34-positive, collagen I, α-smooth muscle actin	
Human, *in-vivo*	Human heart and lung transplant	Sex-mismatched donor lung or heart	No lung cell types of recipient origin	X and Y chromosome FISH, antibody stain for hematopoeitic cells	[36]
Human, *in-vivo*	Human lung transplant	Sex-mismatched donor lung	Bronchial epithelium, type II pneumocytes, glands of recipient origin / 9 – 24%	Y chromosome FISH, short tandem repeat PCR	[35]
	Human BMT	Sex-mismatched donor bone marrow	No lung cell types of donor origin	Y chromosome FISH, short tandem repeat PCR	
Human, *in-vivo*	Human BMT	Sex-mismatched donor bone marrow	Lung epithelium and endothelium of donor origin / up to 43%	X and Y chromosome FISH, keratin and PECAM immunostain	[38]
Human, *in-vivo*	Human BMT	Sex-mismatched donor bone marrow	No nasal epithelium of donor origin	Y chromosome FISH, cytokeratin immunostain	[37]

BMT = bone marrow transplant (with prior ablation), MSC = mesenchymal stem cells (bone marrow stromal cells, adherent bone marrow cells), EGFP = enhanced green fluorescent protein, HSC = hematopoietic stem cells, FISH = fluoresence in situ hybridization, SAEC = small airway epithelial cells

Transplantation studies in mice can be performed using whole donor bone marrow, the fraction that adheres in culture, termed marrow stromal cells (MSC), or preparations enriched for hematopoietic stem cells (HSC). Whole body irradiation, which may injure lung tissue, is typically used to deplete the host bone marrow. Importantly, lung injury apparently enhances engraftment into lung [22-29]. Whole bone marrow, MSC, or HSC have all been reported to reconstitute lung parenchymal cells. MSC transplantation resulted in collagen I expressing donor cells in the lung [30], and in the presence of bleomycin injury, MSC reportedly generated type I [23] or type II pneumocytes [22]. Transplantation with HSCs yielded up to 20% donor-derived pneumocytes and 4% bronchial epithelial cells [31]. However, other investigators have identified only hematopoeitic chimerism by HSCs [32]. Whole bone marrow infusion generated type II pneumocytes [33], or fibroblasts and type I pneumocytes [34]. Radiation pneumonitis augmented whole bone marrow generation of type II pneumocytes and bronchial epithelial cells [25] or fibroblasts [26]. Bleomycin lung injury enhanced formation of type I collagen-producing cells [24] from whole bone marrow, whereas elastase-induced emphysema stimulated formation of alveolar epithelium and endothelium [28]. Lung injury alone, without bone marrow transplantation, may promote stem cell migration. For example, in the ovalbumin model of asthma, circulating fibrocytes were recruited into bronchial tissue [29], and in a bovine model of hypoxic pulmonary hypertension, cells capable of generating endothelial and smooth muscle cells *in vitro* were found in the circulation [27].

Sex-mismatched lung and bone marrow transplantation in humans provides a natural model for analysis of donor and recipient cell behavior. Bronchial epithelial and gland cells and type II pneumocytes of host origin were reported in one study of lung allografts [35], but not another [36]. After bone marrow transplantation, epithelial cells of donor origin were not detected in the nasal passages [37]. Similar to lung allografts, following bone marrow transplantation, epithelium and endothelium of donor origin were found in one study [38], but not another [35].

Many questions remain unanswered. The mechanism whereby cells assume lung cell phenotypes remains uncertain. Several studies have demonstrated that cell fusion occurs both *in vitro* and *in vivo*, which likely explains why some of the cells contain both donor and lung cell markers [see [39] for a study of fusion of MSCs and lung epithelium and [40,41] for recent reviews]. Alternatively, cells may reprogram in the lung environment- a concept termed "transdifferentiation", which is defined as the ability of a particular cell from one tissue type to differentiate into a cell type characteristic of another tissue. It has been

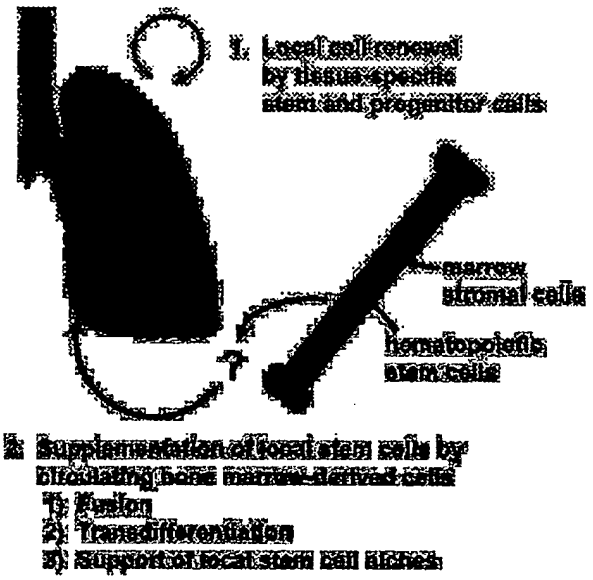

Figure 4

Evolving view of cell lineages in the lungs. The functional significance of circulating cells towards lung cell maintenance or tissue repair remains unknown, as does the precise mechanism whereby circulating cells generate lung cell types.

suggested that many of the events previously attributed to transdifferentiation may actually represent cell fusions, particularly due to the influx of fusion-prone myeloid cells into damaged tissues from the repopulated bone marrow [40]. New, more stringent, criteria have been put forth for demonstration of transdifferentiation [41]. Bone marrow harbors a generalized pluripotent stem cell [42] and the bone marrow cell responsible for lung engraftment has not been identified with certainty. It is possible that rare transdifferentiation events represent migration of a pluripotent bone marrow cell type resembling an embryonic stem or embryonic germ cell still harbored in the adult bone marrow. It remains unknown whether bone marrow cells must transit through an intermediate compartment prior to lung colonization (Figure 4) or whether circulating stem cells can be mobilized from sources other than bone marrow. It is important to note that bone marrow derived cells of typical hematopoietic lineage, chimeric cells created by fusion, or lung cells generated by transdifferentiation may all play a role in lung repair by promoting the local production of stem cells or reparative function of lung-specific cell types. A compelling study suggests that mesenchymal stem cells from bleomycin-resistant mice can mitigate the pro-fibrotic effects of bleomycin in sensitive mice [22], while another study suggests that bone marrow cells actively contribute to the

Table 2: Major lung diseases potentially treatable by stem cell manipulation.

Disease Category	Injured, Depleted, or Deranged Cellular Compartment*	Therapeutic Goals
Congenital lung hypoplasia Chronic lung disease of prematurity Pulmonary emphysema	Alveolar epithelium, Interstitial fibroblast, Capillary endothelium,	Generate alveolar septa Restore complex three dimensional structure
Neonatal RDS Adult RDS	Alveolar epithelium, Capillary endothelium	Enhance surfactant production Reinforce endothelial and epithelial barriers
Pulmonary fibrosis	Alveolar epithelium, Interstitial fibroblast	Prevent alveolar epithelial loss Inhibit fibroblast proliferation
Asthma	Airway epithelium, Myofibroblasts, Airway smooth muscle	Create an anti-inflammatory environment Inhibit airway wall remodeling Inhibit smooth muscle hypertrophy and hyperplasia
Cystic fibrosis	Airway epithelium	Deliver functional CFTR
Bronchiolitis obliterans	Airway epithelium	Reinforce the epithelium against toxic, viral or immunologic injury
Lung cancer	Epithelium	Detection, monitoring or treatment based on molecular regulation of stem cell proliferation and differentiation

RDS = respiratory distress syndrome, CFTR= cystic fibrosis transmembrane conductance regulator *Each cell type listed in this column is affected in all of the specific conditions listed in the left hand column

formation of fibrotic tissue [24]. Mitigating or exacerbating roles for bone marrow derived cells in lung repair or fibrosis are not mutually exclusive. The important concepts of whether the lungs' capacity for repair is dependent on circulating cells, and whether exogenously delivered cells can enhance resistance to injury or promote healing, remain unanswered and controversial.

Lung "stem cell" diseases

Major lung diseases likely involving stem cells and the cellular targets for stem cell therapy are summarized in Table 2. These may be broadly categorized whether they involve stem cell deficiency, hyper-proliferation or possibly, a combination of both. For example, impaired pulmonary endothelial and/or epithelial barrier function may contribute to the pathophysiology of adult respiratory distress syndrome. Mobilization of endogenous endothelial or epithelial stem/progenitor cells or delivery of adult somatic stem cells, embryonic stem cells, or embryonic germ cells may theoretically improve barrier function, supporting the notion of treating a "stem cell deficiency". Similarly, toxic, viral or alloimmune destruction of the bronchiolar epithelium suggests stem cell deficiency in bronchiolitis obliterans. However, fibrotic reactions and scarring in response to epithelial injury can be viewed as fibroblast "stem cell hyper-proliferation". The general concept is that augmentation of stem cells may minimize lung injury, augment repair, or possibly regenerate lost tissue. However, one must also consider that inhibiting excessive growth of stem cells may be a valid therapeutic goal when hyper-proliferation contributes to disease pathophysiology, as in fibrosis, smooth muscle hyperplasia or lung cancer.

Challenges for lung regenerative medicine

What are the realistic prospects for beneficial stem cell therapy of the lung? First, we must conclusively identify lung diseases/cases/timing in which cell and tissue damage occurs in excess of the capacity for timely endogenous repair. Second, we must establish standardized sources of relevant stem/progenitor cells and methods for their delivery to the appropriate lung sub-compartment. Once delivered, therapeutic cells must home to microscopic sites of need and integrate to serve a beneficial function. There is clearly potential for adverse effects, as exemplified by the propensity of embryonic stem cells to form teratomas when implanted *in vivo* [43]. Major lung diseases potentially addressable by stem cell therapy may pose unique challenges. Reversal of lung developmental anomalies resulting in hypoplasia, or repair of chronic lung disease of prematurity and advanced pulmonary emphysema in adults, will require neogenesis of alveolar septa in which the endogenous "tissue blueprint" never developed, or was completely destroyed. Until we gain a much better understanding of lung tissue morphogenesis, we must rely on stem cells intrinsically "knowing" where to go and "how" to recreate alveolar septal architecture to ultimately restore higher order complex three dimensional relationships amongst alveoli, airways, and vessels. Stem cell therapy to cure cystic fibrosis will require heterologous, or gene corrected autologous, stem cells to colonize the airway, proliferate, and differentiate into columnar cells covering a significant portion of the airway lumen. However, most evidence thus far suggests that cells from the circulation may generate isolated, single airway basal cells. Stem cell therapy to mitigate respiratory distress syndrome (RDS) will require cells capable of

restoring alveolar endothelial and epithelial function in the face of evolving injury. Whereas injury is thought to promote stem cell recruitment, the relevant question is whether it can occur quickly enough to meaningfully reverse acute, widespread cellular dysfunction typical of RDS.

Conclusion

Provocative, but controversial, recent evidence suggests that circulating stem cells may home to the lung. There is great excitement and hope that exogenous and/or mobilized endogenous stem cells may be harnessed to prevent or treat acute and chronic lung diseases and even regenerate abnormally developed or lost tissue. Our understanding of lung stem cells and the regulation of lung morphogenesis is still rudimentary, and the complex, integrated function of multiple cell types underlying normal lung structure and function poses unique challenges. Thus, the therapeutic prospects for stem cell therapy in lungs appear more distant than in some other organs. This realization should stimulate meaningful new studies from the lung research community. Unlike the mythical hero Prometheus, patients with lung disease cannot wait 30,000 years!

Competing interests

None declared.

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AB The ex **vivo** expansion of hematopoietic **stem cells** (HSC) for clinical use is now recognized to be a feasible and very promising approach for hematotherapy. Expansion of specific HSC subsets is required for different clinical applications, for example, to increase the number of mature cells, to produce specific cells for adoptive therapy, or to increase the number of primitive **stem cells** available for engraftment. Although hematopoietic **growth** factors can play an important role in this setting, in this **review** we emphasize that other variables affect the outcome of stem and progenitor cell expansion. These variables include the serum supplement, the purity of CD34(+) cells, the initial cell concentration, and the duration of culture. It is also essential to define standard culture conditions for normal **stem cells** and to limit or prevent expansion of residual tumor cells. In clinical applications, determination of the hematopoietic value of the expanded population is mandatory. Thus, we have to demonstrate the expansion of primitive hematopoietic progenitor and **stem cells**, with maintenance of their hematopoietic potential as assessed by in vitro or in **vivo** assays. We draw attention to the **challenges** in the clinical application of ex **vivo** expansion. These include the establishment of well-defined experimental conditions and the determination of the hematopoietic value of the expanded grafts, whatever the graft source: bone marrow, mobilized peripheral blood, or cord blood. Future studies hopefully will optimize these procedures and allow not only expansion but engineering of defined cellular functions as HSCs **grow** under defined conditions.

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TITLE: Adult neural stem cell therapy: expansion in vitro, tracking in **vivo** and clinical transplantation.
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AB Neural **stem cells** (NSCs) are present not only in the developing nervous systems, but also in the adult human central nervous system (CNS). It is long thought that the subventricular zone of the lateral ventricles and the dentate gyrus of the hippocampus are the main sources of human adult NSCs, which are considered to be a reservoir of new neural cells. Recently adult NSCs with potential neural capacity have been isolated from white matter and inferior prefrontal subcortex in the human brain. Rapid advances in the stem cell biology have raised appealing possibilities of replacing damaged or lost neural cells by transplantation of in vitro-expanded **stem cells** and/or their neuronal progeny. However, sources of **stem cells**, large scale expansion, control of the differentiations, and tracking in **vivo** represent formidable **challenges**. In this paper we **review** the characteristics of the adult human NSCs, their potentiality in terms of **proliferation** and differentiation capabilities, as well as their large scale expansion for clinical needs. This **review** focuses on the major advances in brain stem cell-based therapy from the clinical perspective, and summarizes our work in clinical phase I-II trials with autologuous transplantation of adult NSCs for patients with open brain trauma. It also describes multiple approaches to monitor adult human NSCs labeled superparamagnetic nanoparticles after transplantation and explores the intriguing possibility of stem cell transplantation.

nature cell biology

In vivo self-renewing divisions of haematopoietic stem cells are increased in the absence of the early G1-phase inhibitor, $p18^{INK4C}$

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Self-renewal of stem cells is critical for tissue repair and maintenance of organ integrity in most mammalian systems. The relative asymmetry between self-renewal and differentiation in balance with apoptosis determines the size and durability of a stem-cell pool. Regulation of the cell cycle is one of the fundamental mechanisms underlying determination of cell fate. Absence of $p21^{Cip1/Waf1}$, a late G1-phase cyclin-dependent kinase inhibitor (CKI), has previously been shown to enable cell-cycle entry of haematopoietic stem cells, but leads to premature exhaustion of the stem cells under conditions of stress. We show here that deletion of an early G1-phase CKI, $p18^{INK4C}$, results in strikingly improved long-term engraftment, largely by increasing self-renewing divisions of the primitive cells in murine transplant models. Therefore, different CKIs have highly distinct effects on the kinetics of stem cells, possibly because of their active position in the cell cycle, and $p18^{INK4C}$ appears to be a strong inhibitor limiting the potential of stem-cell self-renewal *in vivo*.

Adult stem cells have defined therapeutic roles, as evident in clinical haematopoietic cell transplantation. However, a significant hurdle restricting broader use of adult stem cells is their limited proliferation and favoured differentiation in response to proliferative stimuli, thus compromising *ex vivo* expansion efforts. Therefore strategies to further manipulate the stem cells require greater understanding of their unique ability to self-renew in physiologically determined balance with differentiation or cell death. Regulation of the cell cycle is one of the fundamental mechanisms underlying determination of cell fate, yet molecular events orchestrating its deterministic roles are largely undefined in stem cells. In mammalian cells, entry into the cell cycle requires sequential activation of the cyclin-dependent kinases (CDK) 4/6 and CDK2, which are inhibited by the INK4 proteins ($p16^{INK4A}$, $p15^{INK4B}$,$p18^{INK4C}$ and $p19^{INK4D}$) and the Cip/Kip proteins ($p21^{Cip1/Waf1}$, $p27^{kip1}$ and $p57^{Kip2}$), respectively. Both INK4 and Cip/Kip families compose an important class of cell-cycle inhibitors, termed CDK inhibitors (CKIs)[1]. Whereas a complex array of extracellular signals and intracellular transduction pathways participate in communicating regulatory cues of the cell cycle[2], CKIs appear to be critical mediators of cell-cycle control which may function in a cell-autonomous manner[3–5]. As previously shown in murine haematopoietic cells, p21 deficiency resulted in increased cycling in the haematopoietic stem-cell (HSC) pool, not the committed haematopoietic progenitor-cell (HPC) pool under homeostasis, but stem cell self-renewal potential was compromised in stress conditions[6]. Given that the two CKI families target distinct components in the cell cycle machinery, we hypothesized that the INK4 proteins functioning earlier in G1 may influence the fate of stem-cell division upon mitogenic stimuli in a unique manner. This hypothesis was indirectly supported by recent studies indicating $p16^{INK4A}$and $p19^{ARF}$ as downstream mediators of the Bmi-1 protein regulating HSC self-renewal[7]. The distinct INK4 family member $p18^{INK4C}$ (p18 hereafter) is expressed in multiple tissue types including haematopoietic cells[8], the loss of which in mice results in organomegaly with higher cellularity and increases the incidence of tumorigenesis with advanced age or in the presence of carcinogens[9–11]. We now report an inhibitory role of p18 in divisions of HSCs and the early HPCs through the use of reconstituted mice with p18-deficient haematopoietic cells and extensive *in vivo* evaluation of stem-cell function.

HSCs are responsible for long-term haematopoietic reconstitution of irradiated mice, and their functions can be definitively examined in transplant models. We first took the approach of competitive bone-marrow transplantation (cBMT)[12,13] to directly assess the possible impact of p18 absence on haematopoietic reconstitution (Supplementary Information, Fig. S1). Equal numbers (2×10^6) of bone-marrow nucleated cells from $p18^{+/+}$ mice and $p18^{-/-}$ mice were co-transplanted into lethally irradiated recipients. The relative contribution from each genotype was quantified with a semi-quantitative polymerase chain reaction (PCR) approach[13]. Based on the standardization simultaneously generated under identical PCR conditions (Fig. 1a), $p18^{-/-}$ blood cells constituted 93.3% (versus 6.7% of $p18^{+/+}$ genotype on average) in the mixed populations (Fig. 1b).

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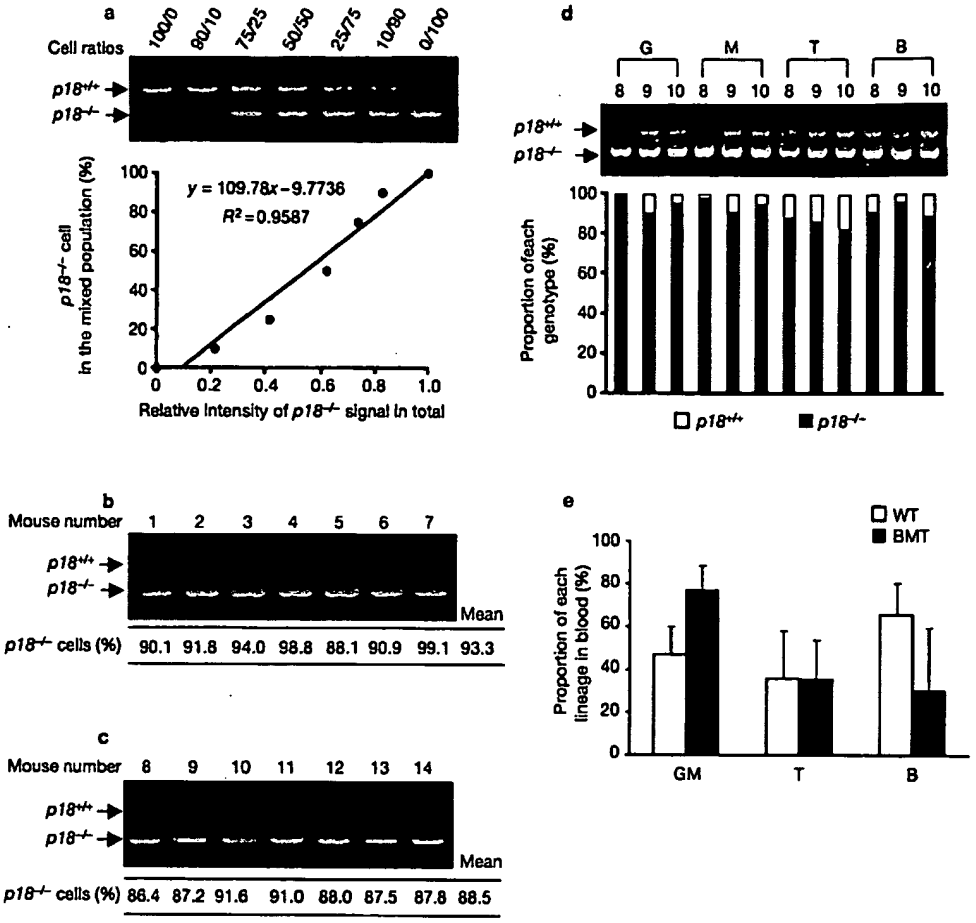

Figure 1 Preferential outgrowth of $p18^{-/-}$ hematopoietic cells during long-term engraftment after primary or secondary cBMT. Bone marrow cells from $p18^{-/-}$ and $p18^{+/+}$ mice were mixed with a 1:1 ratio and injected into lethally irradiated recipient mice (4×10^6 cells per mouse). Bone-marrow cells from the mice at 10 months after primary cBMT were again mixed with freshly isolated bone-marrow cells from non-transplanted wild-type mice at age 8 weeks in a 1:1 ratio and secondarily transplanted into lethally irradiated wild-type recipients (4×10^5 cells in total per mouse). Semi-quantitative PCR was performed at different times to determine the contribution of each genotype to the haematopoietic reconstitution after primary or secondary cBMT. (a) Standardization based on the correlation between the relative density of $p18^{-/-}$ signal in total for each lane on the gel and the actual ratio of the two cell populations. (b) Representative data for blood cells at 7 months after primary cBMT. Labels 1–7 indicate individual recipient mice. According to the standardization, the converted percentages of $p18^{-/-}$ cells in the blood are shown below the PCR gel. (c) Representative data for the blood cells collected at 8 months after secondary cBMT. Lables 8–14 indicate individual recipient mice. (d) $p18^{-/-}$ dominancy in multiple lineages. Marrow cells from the mice (numbers 8, 9 and 10) 12 months after secondary cBMT were stained with lineage markers for myeloid cells (GM: Mac-1$^+$), T cells (T: CD3$^+$) or B cells (B: B220$^+$); each lineage was sorted for genotypic analysis with the semi-quantitative PCR method. (e) Lineage differentiation profile of the transplanted mice (BMT) in comparison and non-transplanted mice (WT). Blood cells were stained with different lineage markers and examined with flow cytometry. GM, T and B indicate lineages for myeloid, T and B cells, respectively. Data shown are mean values from six animals.

Therefore, there was on average a 14-fold greater abundance of the long-term repopulating ability (LTRA) of $p18^{-/-}$ bone-marrow cells compared with the same number of $p18^{+/+}$ marrow cells. To determine whether the increased engraftment of the $p18^{-/-}$ genotype cells occurred at the HSC or different HPC levels, quantitative assays for colony-forming cells (CFCs) (*in vitro* surrogate for lineage-committed HPCs) and long-term culture-initiating cells (LTC-ICs)[14] (*in vitro* surrogate for an HSC subset), were performed with subsequent colony genotypic analyses by PCR. Dramatic overrepresentation of the $p18^{-/-}$ genotype was observed in both the CFC and LTC-IC pools (Table 1). In addition, we found that 91.4% of the Lin$^-$c-kit$^+$Sca-1$^+$ cells (LKS)[15,16] (an *in vivo* immunophenotype enriched for HSCs) were also of the $p18^{-/-}$ genotype 12 months after the primary cBMT (Table 1). These data indicate that $p18^{-/-}$ haematopoietic cells including the primitive subsets have a strong competitive advantage over wild-type cells. It should be noted that we could document no significant difference in bone-marrow homing of p18 null haematopoietic cells to account for the differences in engraftment (data not shown).

To test whether the enhanced engraftment was attributed to increased self-renewal of haematopoietic cells in the absence of p18, serial transplantation was integrated with the cBMT assay (Supplementary Information, Fig. S1). We collected bone marrow cells from mice 10 months after the primary cBMT and performed a secondary cBMT. Bone-marrow nucleated cells from the primarily transplanted mice were re-challenged with an equal amount (2×10^6) of marrow nucleated cells freshly isolated from $p18^{+/+}$ animals at

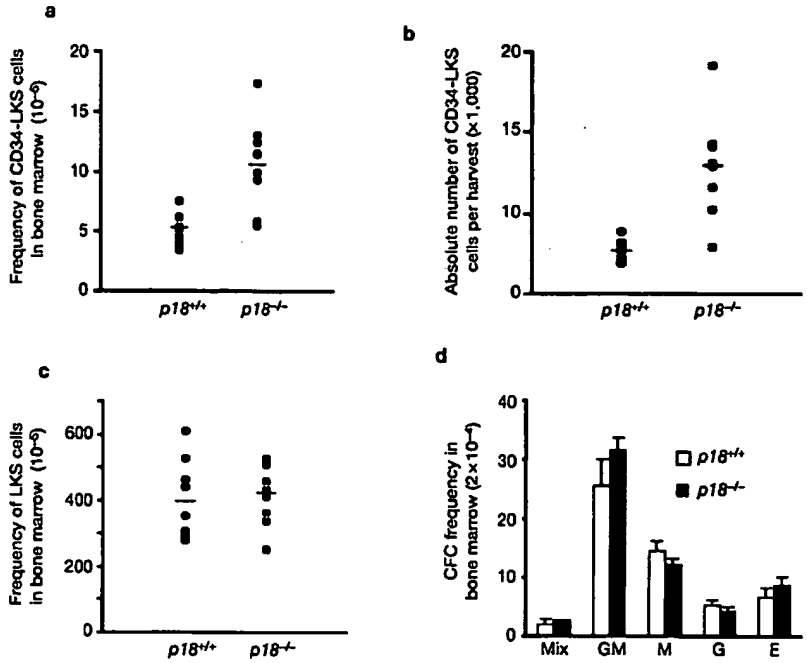

Figure 2 Enlarged pools of HSCs or early HPCs in $p18^{-/-}$ mice under steady-state conditions. (a–c) Phenotypic quantitation for HSCs and committed HPC pools. Bone-marrow nucleated cells from $p18^{-/-}$ mice and gender matched $p18^{+/+}$ mice (8–12 weeks) were analysed by flow cytometry ($n = 9$). HSCs that are negative for lineage markers and CD34, positive for c-Kit and Sca-1, are referred to as 'CD34$^-$LKS' cells (Supplementary Information, Fig. S2a) (ref. 15). LKS$^-$ is the more mature Lin$^-$c-kit$^+$Sca-1$^-$ cells, which are devoid of HSC activity but contain most lineage-committed HPC subsets[20,30]. (d) CFC assay for committed HPCs. Bone-marrow nucleated cells from $p18^{-/-}$ mice and their gender-matched $p18^{+/+}$ littermates were cultured in the defined methycellulose medium supplemented with a cytokine cocktail (see Methods). Mean frequencies and standard deviations of CFCs with different linage characteristics are presented in the graph ($n = 3$). Mix, GM, G, M and E represent CFC-mix (more than two lineages), CFC-granulocyte and monocyte, CFC-granulocyte, CFC-monocyte/macrophage and BFU-erythrocyte, respectively. Student's *t*-test was used to analyse the statistical differences between $p18^{-/-}$ and $p18^{+/+}$ groups. The mean value of the $p18^{-/-}$ group is significantly higher than that of the $p18^{+/+}$ group in a and b ($p < 0.01$), but not in c and d ($p > 0.05$).

Table 1 Follow-up of $p18^{-/-}$ genotype in individual stem/progenitor cells after cBMT.

	Clonal culture	Time after BMT (months)	Number of mice analysed	Total colonies analysed	$p18^{-/-}$ colonies	$p18^{+/+}$ colonies	$p18^{-/-}$ dominance (%)
Primary cBMT							
Experiment 1	CFC	10	3	39	38	1	97.5
Experiment 2	CFC	7	3	122	115	7	94.3
		10	3	108	107	1	99.1
		14	3	144	143	1	99.3
	LTC-IC	10	3	48	45	3	93.7
Experiment 3	CFC	8	3	85	72	13	80.0*
		12	3	122	110	12	90.1*
	LKS	12	2	220	201	19	91.4
Secondary cBMT**							
	CD34$^-$LKS	12 or 22***	3	109	101	8	92.7

Clonal analysis of genotypic origin for the different haematopoietic cells at different differentiation stages or defined by different methods (*in vitro* assays versus immunophenotypes). *The relatively lower percentage was attributed to a lower irradiation rate used in experiment 3. Irradiation dose rate was 5.96 Gy min^{-1} in experiments 1 and 2, and 0.94 Gy min^{-1} in experiment 3 because of the use of different irradiators with unadjustable irradiation dose rates. **Secondary cBMT was performed 10 months after primary cBMT in experiment 2. ***If dated after the primary cBMT.

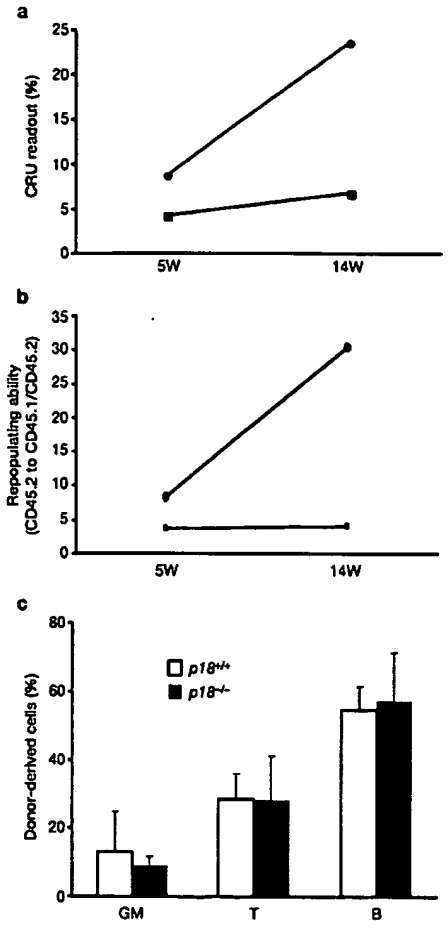

Figure 3 Accelerated repopulation after $p18^{-/-}$ HSC transplantation. According to the experimental design (Supplementary Information, Fig. S2b), different numbers (10, 20 or 40) of $CD34^{-}LKS$ cells ($CD45.2^{+}$) were mixed with 10^5 Sca-1-depleted competitor bone-marrow cells ($CD45.1^{+}/CD45.2^{+}$) and injected into lethally irradiated recipients (CD45.1) ($n = 10$ mice per cell dose). Different lineages in the peripheral blood were analysed 5 and 14 weeks after transplantation. A level of 2.5% or higher of $CD45.2^{+}$ cells associated with multilineage differentiation was defined as positive engraftment in a given animal. CRU values were calculated with L-Calc software. (a) CRU readouts. The graph shows the difference of CRU mean proportions at 5 weeks (5W) and 14 weeks (14W) (see detailed values in Supplementary Information, Table S1). (b) Repopulating ability in the recipients transplanted with a higher dose of HSCs. Eighty $CD34^{-}LKS$ cells were co-transplanted with 10^5 Sca-1-depleted competitor bone-marrow cells into lethally irradiated recipients ($n = 5$). The graph indicates the repopulating ability of the test cells as determined by the ratios of CD45.2 to CD45.1/CD45.2 cells in blood at 5 weeks (5W) and 14 weeks(14W) after transplantation. (c) Multilineage differentiation profile. Multilineage differentiation was examined by flow cytometry. GM, T and B indicate lineages for myeloid, T and B cells , respectively.

8 weeks of age. Strikingly, the $p18^{-/-}$ haematopoietic cells were still able to outcompete the co-transplanted $p18^{+/+}$ cells and became dominant again in the new recipients 8–12 months after the secondary cBMT (Fig. 1c). The LTRA of the $p18^{-/-}$ haematopoietic cells assessed in the secondary recipients remained on average 8-fold greater than that of the $p18^{+/+}$ cells that were newly co-transplanted in the secondary cBMT. To further characterize the breadth of cell types repopulated by the $p18^{-/-}$ cells, immunophenotypically defined cell types from different lineages were sorted from the marrow at 12 months after the secondary cBMT, and tested for the genotypic ratios. Similar to what was found with whole blood cells, the dominance of the $p18^{-/-}$ phenotype was observed in all major types of blood cell (Fig. 1d). Flow cytometric analysis of blood and bone marrow cells from the secondary recipient mice did not reveal predominant growth of a specific lineage compared with the non-transplanted wild type mice (Fig. 1e). These data indicate persistence of regenerated cells with multilineage differentiation potential (HSCs or multipotent HPCs) in the secondary recipients without a leukemic phenotype in these mice.

Stem-cell concentration tends to decrease with serial BMT[17,18] and we previously observed premature exhaustion of HSCs in the absence of p21 (ref. 6). To test whether the $p18^{-/-}$ HSCs manifest the same outcome, we isolated one of the most primitive phenotypes for murine HSCs *in vivo*, the $CD34^{-}LKS$ cells[19] (Supplementary Information, Fig. S2a), from the mice at 12 months after the secondary cBMT and determined their genotypic characteristic at the single-cell level. Among 109 clones from three mice, 92.7% of the $CD34^{-}LKS$ cells were of $p18^{-/-}$ origin (Table 1, bottom line). Therefore, the $p18^{-/-}$ genotype sustains its predominant representation in the HSC pool through nearly two years of serial cBMT without apparent exhaustion. The absence of p18 provides a capacity for increased self-renewal not seen in the absence of the CKI p21 or p27 (refs 6 and 13).

Growth advantage of $p18^{-/-}$ $CD34^{-}LKS$ cells over their wild-type counterparts in the competitive repopulation models suggests a possible expansion of HSCs in the $p18^{-/-}$ non-transplanted mice under homeostatic conditions. This possibility was examined with the phenotypic analysis between littermates or age-matched $p18^{+/+}$ and $p18^{-/-}$ mice with the HSC immuno-phenotype, $CD34^{-}LKS$ (ref. 19) (Fig. S2a). We observed a 2-fold increase in frequency and 3-fold increase in absolute yield per marrow harvest of the $CD34^{-}LKS$ cells in the $p18^{-/-}$ mouse in the C57BL/6;129/Sv background (Fig. 2a, b). In contrast, the more differentiated $Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{-}$ (LKS^{-}) cells, which are devoid of HSC activity but contain most lineage-committed HPC subsets[20], had an insignificant change in frequency (Fig. 2c). These data were highly consistent, with no difference in the frequency of different CFCs from unfractioned bone-marrow nucleated cells between the two genotypes (Fig. 2d). Therefore, HSCs but not the late lineage-committed HPCs appeared to increase in the absence of p18.

A 2-fold increase of HSC frequency ($CD34^{-}LKS$) in $p18^{-/-}$ bone marrow was thought to be insufficient to account for the dramatic engrafting advantage of the $p18^{-/-}$ cells over the $p18^{+/+}$ cells after the subsequent cBMT (Fig. 1b, c). Rather, ongoing regeneration of 18–/– HSCs after transplantation was considered more likely. To further define this issue, we performed stem-cell transplantation to assay the competitive repopulation units (CRUs) with limiting dilution analysis[26] in the defined stem-cell population ($CD34^{-}LKS$ cells). To this end, the original C57BL/6;129/Sv strain was backcrossed into the pure C57BL/6-Ly5.2 (CD45.2) background for 10 generations, allowing us to accomplish a quantitative analysis with the experiment in congenic mouse strains (CD45.1 as recipients and CD45.1/CD45.2 F1 as competitor cells) (Supplementary Information, Fig. S2b). Doubly sorted $CD34^{-}LKS$ cells from $p18^{-/-}$ or $p18^{+/+}$ 8-week-old mice in the background (C57BL/6-CD45.2) were used as donor cells for measuring their CRUs (10, 20 or 40 $CD34^{-}LKS$ cell per mouse and 10 mice per dose). We collected blood and stained the distinct congenic markers for CD45 antigen (CD45.1 versus CD45.2 versus CD45.1/CD45.2) at weeks 5 and 14 after transplantation to calculate the CRU frequency (Supplementary Information, Table S1). Interestingly, although CRU

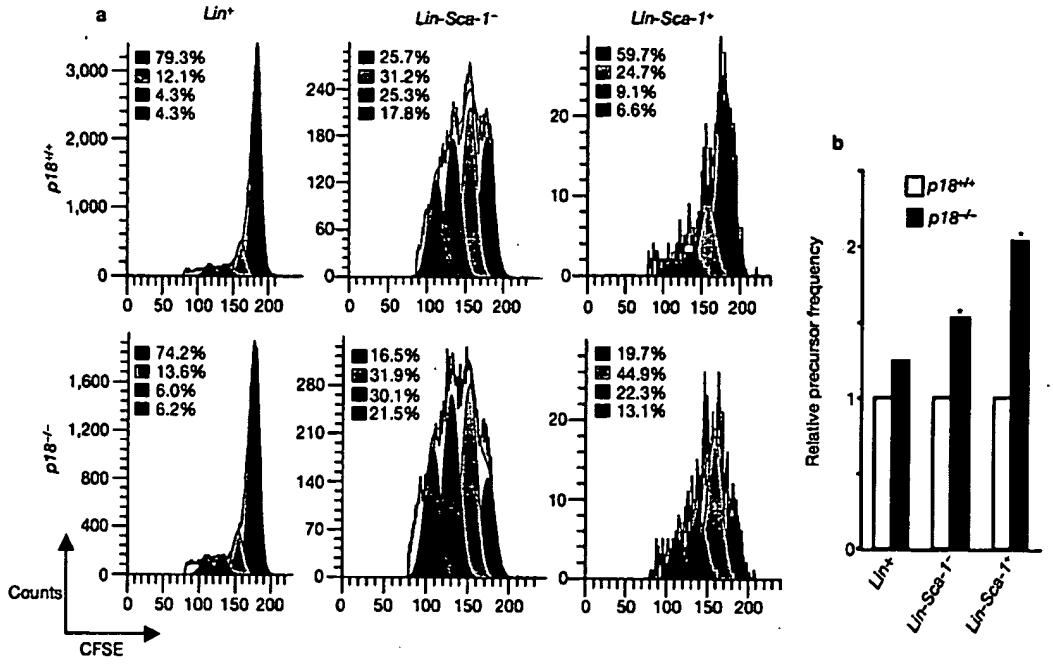

Figure 4 Favoured primitive phenotype after divisions of $p18^{-/-}$ haematopoietic cells *in vivo*. Bone marrow cells were labelled with CFSE, injected into lethally irradiated recipient mice and harvested 2 days after the transplantation to assess the number of cell divisions. Cells were stained with the lineage and stem-cell markers described in Methods. CFSE-labelled cells were analysed in the gate for a specific phenotype. (a) Representative figure of the flow cytometric analysis. Blue peaks on the right indicate undivided cells (parent cells) and each peak towards the left-hand side represents one cell division or generation. The percentages of the cells in each division obtained in a representative experiment are inserted in the graphs. The figure shown is from one of four experiments with similar results. (b) Summary of the mean values from four independent experiments. An assumption made in the computation model is that cell number will double as cells proliferate through each daughter generation in a given population (Lin^+ versus $Lin^-Sca\text{-}1^-$ versus $Lin^-Sca\text{-}1^+$). ModFit LT software was used to calculate 'precursor frequency', the proportion of the total cells calculated to have been present at the start of the experiment (derived by back-calculation according to the model) that had gone on to true proliferation during the course of cell division. Data shown are the ratios of the precursor frequency between $p18^{-/-}$ and $p18^{+/+}$ cell populations (four experiments, three to five donor mice for each genotype in each experiment). $^*p < 0.05$.

readout modestly increased from 4.25% to 8.58% in $p18^{+/+}$ $CD34^-LKS$ cells, it substantially increased from 6.82% to 23.59% in $p18^{-/-}$ $CD34^-$ LKS cells (Fig. 3a), thereby indicating a great enrichment for the HSCs being transplanted in both groups and, moreover, a higher repopulating rate of the *p18-/-* HSCs. Normalized for the CRU frequency obtained at 14 weeks after transplantation (a more relevant and standard time for measuring HSCs) and abundance of $CD34^-LKS$ cells in the marrow (Fig. 2a, b), there was approximately a 7-fold increase in frequency and a 10-fold increase in absolute yield (two femurs and two tibias) of CRU in the $p18^{-/-}$ bone marrow. Further, as an independent assessment for the increased regeneration of $p18^{-/-}$ HSCs after transplantation, we then transplanted different groups of mice (five mice each group) with a saturating dose of 80 $CD34^-LKS$ cells per mouse plus the competitor cells. Interestingly, there was also a 7-fold increase of relative engraftment level compared with competitor cells in $p18^{-/-}$ groups 3 months post-transplant without apparent alteration in lineage differentiation ratios (Fig. 3b, d). More interestingly, these data concur with the 14-fold increase in $p18^{-/-}$ LTRA by the cBMT model (Fig. 1b) if normalized for the 2-fold increase of $CD34^-LKS$ cells in the unfractioned marrow.

To directly obtain evidence whether p18 absence may favour primitive phenotypes after each cell division *in vivo*, we next measured cell divisions in distinct immunophenotypically defined cell populations among donor cells in irradiated recipients after BMT. The dye, 5- (and 6-) carboxy-fluorescein diacetate succinimidyl ester (CFSE)[21], was used to label the donor cells before tail injection, and surface markers for HSCs and HPCs were applied to co-stain the marrow cells harvested 2 days after BMT. The number of initial cell divisions was measured based on the intensity of CFSE in each cell population in the recipients. Owing to the undetectable level of c-kit expression (H. Shen, Y. Yuan and T. Cheng, unpublished observations) and up-regulation of CD34 antigen on the donor cells shortly after transplantation[22], we were not able to use the $CD34^-LKS$ or LKS phenotype in these experiments. Instead, Sca-1 was used to define a relatively primitive state within the Lin^- cell population. Within three cell divisions detected in the experiment, there was a significant increase of the cells that divided and retained the same phenotype in both $p18^{-/-}$ $Lin^-Sca\text{-}1^+$ and $p18^{-/-}$ $Lin^-Sca\text{-}1^-$ parent populations compared with the $p18^{+/+}$ controls (measured as 'precursor frequency' in flow cytometry). However, among the $p18^{-/-}$ cells, the increase of cell division seen in the $Lin^-Sca\text{-}1^+$ cell subset was approximately 2-fold more than that seen in the HPC-dominated $Lin^-Sca\text{-}1^-$ cell subset (Fig. 4). As expected, only a small portion of cells was divided in the more differentiated and fully mature (Lin^+) cells, most cells in the marrow and, notably, there was no increased proliferation of $p18^{-/-}$ Lin^+ cells. In agreement with the phenotypic analysis of the haematopoietic cells (Fig. 2), these data also suggest a selective effect of p18 absence on the primitive cells than majority committed HPCs. An independent

assessment using bromodeoxyuridine (BrdU) incorporation in either transplanted or non-transplanted mice further confirmed no general increase of cell proliferation in most committed HPCs and intermediate downstream cells in the absence of p18 (data not shown). In addition, co-staining of the apoptotic marker, Annexin V, in the CFSE$^+$ homed cell populations demonstrated slightly increased apoptotic fractions in $p18^{-/-}$ Lin$^-$ cells compared with $p18^{+/+}$ Lin$^-$ cells in the irradiated recipients (Supplementary Information, Fig. S3), indicating that there was no more prolonged survival that may account for the increase of the $p18^{-/-}$ primitive cells.

Our current study demonstrates that the absence of p18 causes enhanced potential of self-renewal leading to an expansion of HSCs and multipotent HPCs. In the setting of transplantation, p18-deficient cells propagate and allow sequential transplantation beyond that of wild-type cells. Unlike previous demonstrations in p27-deficient mice, in which the pool size and cell cycling of committed HPCs but not HSCs are markedly increased[13], the engraftment advantage in the absence of p18 is not due to predominant outgrowth of a single lineage or less restricted cell cycling in committed HPC pools and more differentiated cell populations. Therefore, our data suggest a relatively specific effect of p18 loss on HSCs and early HPCs.

Although both p21 and p18 appear to affect kinetics in HSCs, they have very distinct phenotypes: p21$^{-/-}$ HSCs undergo premature exhaustion[6] whereas $p18^{-/-}$ HSCs self-renew. Without overwhelming non-specific proliferation in other cell populations, increased regeneration of $p18^{-/-}$ HSCs suggests that the balance of differentiation to self-renewal in the absence of p18 favours self-renewal. This notion is indirectly supported by the data from others demonstrating that p18-expressing cells have an increase in asymmetric division[4,23]. The plausible mechanism of favoured HSC symmetric division may also be associated with the increase of the absolute rate of HSC cycling, which we cannot rule out based on the current data. But the net HSC expansion would argue against the latter possibility as a sole mechanism for the increased HSC self-renewal. It is believed that critical decisions of cell fate are made during G1 (ref. 24). Upon mitogenic stimuli, cyclin D is upregulated and interacts with CDK4/6, resulting in Rb phosphorylation to initiate cell cycle progression. Although Cip/Kip proteins (such as p21) broadly inhibit CDK2 in late G1/S and possibly CDK1 in M phase, they are not capable of inhibiting CDK4/6 activity early in G1 (ref. 25). In contrast, INK4 proteins (such as p18) are able to specifically compete with cyclin D to bind CDK4/6 in early G1 (ref. 26). Given the distinct effects of these two CKI families in stem-cell regulation[6,7,13], we propose a model in which modulation of a distinct CKI or its class at a specific position of the cell cycle may be an important mechanism for balancing self-renewal and differentiation in stem cells. In this model, p18 appears to be a CDK4/6 inhibitor with a unique role in governing HSC self-renewal. Furthermore, the alteration in the overall organ size of the $p18^{-/-}$ mouse[9] indicates that the impact of p18 on primitive cells may not be restricted to haematopoietic tissues. In the absence of p18, stem-cell expansion may be more readily achieved and, therefore, down-modulating p18 may allow enhanced stem-cell expansion, a hypothesis that can now be tested in human adult stem cells *ex vivo*. □

METHODS

Mice. $p18^{+/-}$ mice in a C57BL/6;129/Sv background were imported from the laboratory of David Franklin at Purdue University[9]. $p18^{-/-}$ or $p18^{+/+}$ mice were generated from $p18^{+/-}$ breeding pairs. Mouse colonies were maintained in the certified animal facility at University of Pittsburgh Cancer Institute. Mice were genotyped by a PCR approach using the tail DNA (primers described below). Littermates or age-matched mice (8–12 weeks) were used in competitive BMT and stem-cell phenotypic analysis. For transplantation with purified stem cells and CRU analysis, the mice with the mixed background were bred back into C57BL/6-Ly5.2 (CD45.2) background for 10 generations. Wild-type recipients for transplantation in the B6129/SvF1 background or B6.SJL-Ly5.1 (CD45.1) congenic background were purchased from Jackson laboratory (Bar Harbor, ME). All procedures involved in the mouse work were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

cBMT coupled with serial transfer. Equal numbers of bone-marrow nucleated cells (2×10^6 each) from $p18^{+/+}$ and $p18^{-/-}$ mice were mixed and transplanted into the recipients, which were treated with 10 Gy whole-body irradiation at the rate of 5.96 Gy min^{-1} or 0.94 Gy min^{-1} depending on the configuration of a specific ^{137}caesium irradiator used in different experiments. To perform the secondary cBMT, bone-marrow cells were harvested from the mice 10 months after the primary cBMT, mixed with freshly isolated wild-type bone-marrow cells (non-transplanted cells from mice at age 8 weeks) at a 1:1 ratio, and secondarily transplanted into new lethally irradiated wild-type recipients (age 8 weeks). Blood from the transplanted mice was collected at different times for genotypic analysis with the semi-quantitative PCR method. At varied times after the primary or secondary cBMT, some mice were sacrificed and bone-marrow nucleated cells were used for genotypic analysis in different lineages and HSC or HPC compartments involving the single-cell or colony assays.

Semi-quantitative or single-colony PCR. The contribution of $p18^{+/+}$ or $p18^{-/-}$ cells was determined by semi-quantitative PCR with the following three primers: p18WT-F (5'-AGCCATCAAATTTATTCATGTTGCAGG-3'), P18MG-47-R (5'- CCTCCATCAGGCTAATGACC-3'), and PGKNEO-R (5'-CCAGC-CTCTGAGCCCAGAAAGCGAAGG-3'). The spleen cells from $p18^{+/+}$ and $p18^{-/-}$ mice were mixed at different ratios for standardization of the PCR reaction. For single-colony PCR, individual colonies were picked up with micromanipulation and lysed in 1× PCR buffer containing 2.5 mM $MgCl_2$ and 100 μg ml^{-1} Proteinase K for 1 h at 60 °C, followed by inactivation of the reaction for 20 min at 95 °C.

CFC assay. Freshly isolated bone-marrow nucleated cells were placed in the defined methycellulose medium M3434 (Stem Cell Technologies) supplemented with a cocktail of recombinant cytokines including murine stem-cell factor (50 ng ml^{-1}), murine interleukine-3 (20 ng ml^{-1}), human interleukine-6 (20 ng ml^{-1}) and human erythropoietin (2.5 U ml^{-1}). Cells were plated in six-well plate with 2-ml volume at a density of (1–2) × 10^4 cells ml^{-1}. The CFC colonies were then scored at day 7–14 under an inverted microscope and reported as total colony yield or colony number in a specific lineage. For genotypic analysis in the recipients of cBMT, colonies were picked up and assayed for the p18 genotype with PCR.

LTC-IC culture. Bone-marrow long-term culture was performed as previously described[6] with minor modifications. Briefly, the unfractioned bone-marrow cells were plated on an irradiated (15 Gy) primary mouse stromal monolayer in 96-well plates containing 150 μl of M5300 medium (Stem Cell Technologies) supplemented with 10^{-6} M hydrocortisone. For genotyping analysis in the mice after cBMT, sufficient wells at the limiting dose of approximately one LTC-IC[14] per well were included. The medium was changed with half fresh medium weekly and the long-term culture at week 5 was overlaid with 100 μl of M3434 (Stem Cell Technologies). The plates were evaluated for the presence of CFC colonies at 10 days. The colonies were microisolated and followed by PCR analysis for the p18 genotype.

Flow cytometric analysis. For stem-cell quantitation, the bone-marrow nucleated cells were stained with a mixture of biotinylated antibodies against mouse CD3 (CT-CD3), CD4 (CT-CD4), CD8 (CT-CD8a), B220 (RA3-6B2), Gr-1 (RB6-8C5), Mac-1 (M1/70.15) and TER-119 (Caltag), then co-stained with streptavidin-PE-Cy7, anti-Sca-1-PE (D7), anti-c-Kit-APC (2B8) and anti-CD34-FITC (RAM34) (BD PharMingen). Propidium iodide was used for dead-cell discrimination. A MoFlo High-Speed Cell Sorter (DakoCytomation) and the Summit software (version 3.1, DakoCytomation) were used for data acquisition and analysis. For lineage phenotypic analysis in transplanted mice, 50 μl of the blood was stained with anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD3

(145-2C11), anti-B220 (RA3-6B2), anti-MAC-1-(M1/70) and anti-Gr-1(RB6-8C5) (BD PharMingen). The red cells were lysed with FACS Lysing Solution (BD Biosciences) and analysed by a CyAn (DakoCytomation) or Beckman-Coulter XL (Beckman Coulter) cytometer.

Single stem-cell sorting and culture. The Sca-1$^+$ cells were isolated from bone marrow cells by using the EasySpe kit according to the manufacturer's protocol (StemCell Technologies) and then stained with the mixture of lineage-specific antibodies listed above, anti-c-kit-APC and anti-CD34-FITC. LKS or CD34$^-$ LKS cells were sorted into 384-well plates (Nunc) at one cell per well using the MoFlo High-Speed Cell Sorter with subsystems of CyCLONE Automated Cloner and SortMaster Droplet Control. Each well contained 50 μl of IMDM supplemented with 50 ng ml^{-1} Flt3 ligand (Flt3-L), 50 ng ml^{-1} SCF and 10 ng ml^{-1} TPO. After culture for 14 days, the morphology of each colony was examined under a microscope and the colonies were lysed for PCR.

Stem cell transplantation. Sorted CD34$^-$LKS cells from *p18*$^{-/-}$ or $^{+/+}$ 8-week-old mice in the background of C57BL/6 (CD45.2) (three mice for each genotype) were used for measuring the competitive repopulating unit (CRU). CD34$^-$LKS cells at a limiting dose (40, 20 or 10 cells per mouse) were mixed with 10^5 Sca-1-depleted[27] bone-marrow cells from F1 mice of C57BL/6 and B6.SJL (CD45.1$^+$ and CD45.2$^+$). The cell suspension was injected through tails into B6.SJL (CD45.1$^+$) mice that were irradiated at a fractioned dose of 11 Gy. Ten recipients were included for each group at each dose. Blood cells from the recipients were stained with PE-CD45.1 and FITC-CD45.2 to determine engraftment level of donor cells after transplantation. In a given animal, 2.5% or higher of CD45$^+$ cells containing granulocytes, monocytes and lymphocytes was defined as positive engraftment. The Beckman-Coulter XL cytometer was used for data acquisition. Based on the Poisson distribution of the negatively engrafted mice, CRU values were calculated with L-Calc software (Stem Cell Technologies)[28]. Animals that died during the course were not counted in the limiting dilution analysis, according to the protocol established by others[29]. Detailed parameters and data about the CRU assay are summarized in Supplementary Information, Table S1. As an independent test to determine the engraftment levels, an additional five recipients for each group were transplanted with a higher dose of CD34$^-$LKS cells (80 cells per mouse).

In vivo assay for tracking cell divisions. Bone-marrow cells were labelled with 1 μM of CFSE (Molecular Probes) as described[21]. CFSE-labelled *p18*$^{+/+}$ or *p18*$^{-/-}$ bone-marrow cells (10^8) were injected into a lethally irradiated mouse. Two days after transplantation, recipient marrow cells were stained with the antibody cocktail for lineage markers Sca-1 and c-Kit. The MoFlo High-Speed Cell Sorter was used for data acquisition, and ModFit LT software (version 3.0, Verity Software House) was used for analysis of cell proliferation.

Statistical analysis. Student's *t*-test was used to analyse the statistical differences between *p18*$^{-/-}$ and *p18*$^{+/+}$ groups, with the *p*-values indicated in the related graphs.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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Genetic programs regulating HSC specification, maintenance and expansion

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All mature blood cells originate from a small population of self-renewing pluripotent hematopoietic stem cells (HSCs). The capacity to self-renew characterizes all stem cells, whether normal or neoplastic. Interestingly, recent studies suggest that self-renewal is essential for tumor cell maintenance, implicating that this process has therapeutic relevance. Unfortunately, the molecular bases for self-renewal of vertebrate cells remain poorly defined. This article will focus on the developmental mechanisms underlying fetal and adult HSC homeostasis. Specifically, distinctions between genetic programs regulating HSC specification (identity), self-renewal (in both fetal and adult) and differentiation/commitment will be discussed with a special emphasis on transcriptional and chromatin regulators.
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Self-renewal: a defining property of the hematopoietic stem cell?

The hematopoietic stem cell (HSC) can be operationally defined as a long-term repopulating cell with both lymphoid (T and B) and myeloid potential (reviewed in Orlic and Bodine, 1994). The first evidence for the existence of such a cell-type came from experiments by Ray Owen and co-workers, in 1945, which showed that bovine fraternal twins, sharing a single placenta and blood circulation, retained production of blood cells genetically defined to be from both throughout their life (Owen, 1945). Twenty years later, elegant experiments by Till, McCulloch, Wu, Becker, Siminovitch and co-workers demonstrated that adult bone marrow contained single cells that had the ability to form macroscopic nodules of myeloerythroid cells on the spleen, 8–12 days after intravenous injection into myeloablated recipients (Becker *et al.*, 1963; Siminovitch *et al.*, 1963). These spleen-colony-forming-units (CFU-Ss) were shown to be clonal (Wu *et al.*, 1968a, b) and, in many cases, could generate similar colonies upon transplantation into secondary recipients (Siminovitch *et al.*, 1963). As they shared several characteristics attributed to HSCs (including high proliferative potential, multipotentiality and self-renewal ability), CFU-S were initially considered to be HSCs (Siminovitch *et al.*, 1963). The validity of the CFU-S assay to detect HSCs with long-term repopulating potential was questioned after the discovery that some of these cells were capable of only unilineage differentiation and/or lacked the ability to self-renew (functional heterogeneity). Although most of the cells possessed the ability to differentiate into the erythrocyte and myeloid lineages, their lymphoid potential remained controversial (Wu *et al.*, 1968a, b; Lala and Johnson, 1978; Lepault *et al.*, 1993). It is now clear that most CFU-S cells in the adult bone marrow are committed myeloid progenitors (Worton *et al.*, 1969; Jones *et al.*, 1989), which can be physically separated from more primitive cells with long-term lympho-myeloid repopulating potential (Mulder and Visser, 1987; Visser and de Vries, 1988; Jones *et al.*, 1990; Spangrude *et al.*, 1991; van der Loo *et al.*, 1994). Although the CFU-S assay played a key role in the development of concepts of primitive hematopoietic cell organization and regulation, its inability to analyse pure stem cells meant that most of their functions were implied rather than directly analysed.

The first attempts at purifying the HSC came from experiments carried out by Till and McCullogh (Worton *et al.*, 1969; van Bekkum *et al.*, 1979) in the Netherlands. From this work, it has become possible to routinely identify and isolate highly purified murine and human HSCs based mainly on characteristic cell surface proteins that are either present (Sca-1 and c-kit) or absent (using markers of lineage committed cells such as CD38, Mac-1 and CD8) (reviewed in Weissman, 2002).

Despite the progress that has been made in identifying and obtaining enriched HSC populations, analysis of the population dynamics and cell cycle kinetics of HSCs remains difficult. One of the most intriguing properties of adult HSCs is a robust maintenance of the dynamic equilibrium between self-renewal and differentiation

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(Morrison *et al.*, 1997). Under homeostatic conditions *in vivo*, most HSCs are quiescent, as demonstrated by their relative resistance to killing by the cytotoxic drug 5-fluorouracil (5-FU) when compared to committed progenitor cells (Hodgson and Bradley, 1979; Lerner and Harrison, 1990). When they enter cycle, HSCs can divide asymmetrically or symmetrically, resulting in different HSC fates (see Faubert *et al.*, in this issue). Stem cell maintenance divisions give rise to one daughter HSC with essentially identical biological properties (a process referred to as self-renewal) and one committed daughter cell. The committed daughter cell enters a transient state of rapid cellular proliferation and, upon exhaustion of its proliferative potential, withdraws from the cell cycle and progressively acquires the specialized characteristics of a predetermined blood cell-type. Although the relative influence of intrinsic versus extrinsic factors on HSC self-renewal remains to be determined, it has been easier to identify the environmental factors having a negative impact on this process than those that enhance it. Thus, most *in vivo* culture conditions defined to date lead to depletion of the HSC pool by favoring symmetric divisions (generation of two daughter differentiated cells) and concomitant expansion of committed progenitor populations (Morrison *et al.*, 1997; see also Sauvageau, Iscove and Humphries, in this issue).

Several studies using retroviral marking have demonstrated the ability of HSCs to undergo self-renewal divisions (Lemischka *et al.*, 1986; Jordan and Lemischka, 1990; Keller and Snodgrass, 1990; Fraser *et al.*, 1992). Although most of these studies failed to accurately quantify the magnitude of self-renewal events, considerable evidence suggests that this property is not unlimited (see Bell and van Zant, in this issue). First, following bone marrow transplantation, the HSC pool is not found to regenerate to levels higher than 10% of normal pretransplantation values, despite a complete regeneration of bone marrow cellularity and progenitor cell numbers (Harrison *et al.*, 1978, 1990; Harrison and Astle, 1982; Mauch and Hellman, 1989; Pawliuk *et al.*, 1996). Some investigators have suggested the involvement of negative feedback mechanisms imposed *in vivo* by more mature cells as a possible mechanism that could prematurely inhibit HSC expansion following transplantation (Iscove and Nawa, 1997). Alternatively, this loss of long-term repopulating ability may result from damage to the recipient's microenvironment inflicted by the conditioning regimen (i.e. irradiation). However, experiments performed in the anemic (WW^v) recipient mouse strain, which possesses a normal microenvironment but poorly competitive hematopoietic cells due to a mutation in the *c-kit* ligand receptor (Chabot *et al.*, 1988), rather suggest that this defect is intrinsic to the transplanted cells themselves (Harrison and Astle, 1982; Gardner *et al.*, 1988).

A major concern is that nearly all HSC assays assessing self-renewal rely on the generation of functionally mature cells, and therefore provide a retrospective rather than a current view of potential HSC attributes. In a transplantation setting, the accuracy of the HSC readout relies on the efficiency of the transplanted cells to home and engraft to the specialized niches of the bone marrow microenvironment (Benveniste *et al.*, 2003). The heterogeneity of the HSC compartment further complicates the interpretation of such experimental designs. Age-related and strain-specific (Van Zant *et al.*, 1991; Phillips *et al.*, 1992) differences in HSC numbers and/or competitive abilities have been reported (reviewed in Geiger and Van Zant (2002). Moreover, a functional decline in the proliferative potential of HSCs derived from the fetal liver, umbilical cord (at birth) and adult bone marrow indicates ontogeny-related differences in HSC function (Pawliuk *et al.*, 1996; Rebel *et al.*, 1996a, b; Harrison *et al.*, 1997). Whether this heterogeneity represents true intrinsic quantitative and/or qualitative differences in HSC properties, or in the expression of this potential due to stochastic events, remains unclear.

Genetic programs specifying HSCs

At least three distinct genetic programs are required for the functionality of the blood system. These include: (a) the specification of HSCs, (b) their self-renewal and (c) their commitment–proliferation–differentiation (Figure 1). Several genes regulating HSC specification (Figure 1a) or differentiation (Figure 1c) have been identified (reviewed in Cantor and Orkin, 2001; Kondo *et al.*, 2003) but, as discussed below, determinants of HSC self-renewal remain poorly characterized (Figure 1b). In the next sections, we will review the role of key nuclear factors underlying the molecular programs of HSC specification, self-renewal expansion (SR-E) and maintenance (SR-M).

Genes specifying HSCs

During mouse ontogeny, the first bloods cells, embryonic (or primitive) erythrocytes, arise within the blood

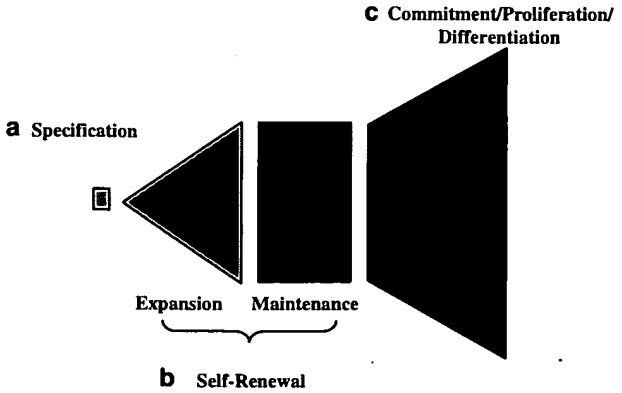

Figure 1 Genetic programs in hematopoiesis. At least three genetic programs are required for the development of the blood system. (a) The specification of HSCs (yellow) (genesis), (b) the self-renewal, including expansion (orange) and maintenance (red) of HSCs as described in Figure 4 and (c) the commitment, proliferation and differentiation of HSCs (dark red)

islands of the extraembryonic yolk sac at embryonic day 7.5 (E7.5). By E11.5, hematopoiesis shifts to the fetal liver, where adult (or definitive) red cells, as well as cells of other hematopoietic lineages, first appear. The site of origin of the HSCs has been less certain. Whereas it was previously accepted that HSCs and progenitors migrate from the yolk sac to the fetal liver during development, more recent studies relying on cell transplantation to reconstitute hematopoiesis in adult recipients assign an intraembryonic source for definitive (adult) hematopoiesis within the intraembryonic para-aortic splanchnopleura (PS) and aortic-gonadal-mesonephros (AGM) regions (Godin *et al.*, 1993; Medvinsky *et al.*, 1993; Medvinsky and Dzierzak, 1996). HSCs arising in these areas are believed to migrate to and colonize the fetal liver and spleen, where they continue to differentiate into recognizable hematopoietic precursors. After birth, definitive hematopoiesis is primarily confined to the bone marrow, and in some pathological conditions, also to extramedullary sites such as the spleen, the liver and occasionally the lung and brain. The presence of multipotential progenitors in the blood of E10 embryos suggests that migration and colonization are mediated via the circulation (Delassus and Cumano, 1996). A unique origin of HSCs is challenged by recent evidence demonstrating long-term repopulation by yolk sac (extra-embryonic) progenitors as assayed by reconstitution of fetal recipient animals (Yoder *et al.*, 1997). Hence, the origin of adult hematopoietic cells (and in particular definitive HSCs) within the specific vascular regions of the mammalian embryo body remains highly speculative. Nevertheless, the development of a stable functioning hematopoietic system reflexes complex processes involving cellular differentiation, as well as temporal and spatial control of migration, homing, self-renewal/proliferation and survival of HSCs.

The transcriptional machinery governing early HSC function is undoubtedly very complex. Genes involved in specifying HSCs during early embryogenesis include: *SCL/tal-1* (Shivdasani *et al.*, 1995) (*stem cell leukemia hematopoietic transcription factor*) and *Rbtn2* (Warren *et al.*, 1994) (also known as *Lmo-2* or *ttg-2*), which are essential for primitive and definitive hematopoiesis and *AML-1* (Okuda *et al.*, 1996; Lacaud *et al.*, 2002) (also known as *RUNX1/CBFA2 and PEBP2B*), that is specifically required for definitive hematopoiesis. Other factors appear to be more lineage-specific in action such as *GATA-3, Ikaros, PU.1, GATA-1, CBP, Atf4, c-myb, T-bet* and *E2A*, as their absence affects specific hematopoietic lineages (see Table 1).

The b-HLH *SCL/tal-1* transcription factor is essential for the establishment of primitive hematopoiesis. Mice lacking *SCL/tal-1* are embryonic lethal and show an absence of yolk sac-derived hematopoiesis (Robb *et al.*, 1995, 1996; Shivdasani *et al.*, 1995; Porcher *et al.*, 1996). Using a conditional gene targeting approach, Orkin and co-workers recently established that *SCL1/tal-1* is critical for the genesis of HSCs, but that its continued expression is dispensable for HSC function (Mikkola *et al.*, 2003). This suggests that distinct classes of HSC regulatory factors may exist: those required for their genesis (i.e. identity), and those specifically required for later functions, such as long-term repopulating activity (self-renewal) and multipotency (see below).

The LIM domain-containing Rbtn2/Lmo-2 protein (which is known to form a heterocomplex together with SCL/tal-1) (Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994) also acts very early in ontogeny, as mice deficient for this gene lack all lineages of both primitive and definitive hematopoiesis, although yolk sac-derived macrophages have been observed (Warren *et al.*, 1994) (see Table 1).

Mice nullizygous for *AML-1* are embryonic lethal and lack fetal liver-derived (definitive) hematopoiesis. However, primitive hematopoiesis is not affected since large nucleated erythrocytes are present in the embryo. *AML-1*$^{-/-}$ ES cells fail to contribute to adult hematopoietic tissues in chimeras (Okuda *et al.*, 1996; Wang *et al.*, 1996a, b, c). Inducible gene-targeting experiments demonstrated that *AML-1* is dispensable for adult HSC function (Ichikawa *et al.*, 2004). *AML-1* appears to be required for the proper temporal and spatial localization of stem cells in the embryo (Cai *et al.*, 2000). Deletion of *Cbfβ*, which enhances the binding activity of *AML-1 in vivo*, is also embryonic lethal. Surprisingly, its effect on fetal liver hematopoiesis is less drastic, suggesting that AML-1 can operate in part without Cbfβ (Sasaki *et al.*, 1996; Wang *et al.*, 1996a, b, c). Examination of the contribution of these genetic axes to later HSC properties (such as HSC self-renewal, proliferation and multipotency) is eagerly awaited.

Intrinsic regulators of fetal HSC self-renewal

After their specification early in ontogeny (see above and Yoder in this issue), HSCs undergo two rounds of mobilization: first, to the embryonic fetal liver where they expand and second, to the bone marrow where they are maintained throughout adult life. Expansion in the fetal liver implies symmetrical HSC divisions referred herein as 'SR-E' for self-renewal expansion. HSC maintenance in the bone marrow suggests asymmetrical divisions named hereafter 'SR-M' for self-renewal maintenance (see next section and Figure 2). Although not proven yet, evidence suggests that these two developmental processes may be intrinsically regulated by distinct sets of genetic determinants.

Embryonic/fetal liver HSC SR-E is necessary to increase the pool size of the mobilized stem cell population. HSCs deficient in the genetic program necessary for SR-E will not expand, but depending on the intensity of the defect, they may generate enough mature blood elements to ensure the viability of an animal. This hypothesis is supported by serial transplantation experiments, which indicated that hematopoietic mouse chimeras reconstituted with as few as 280 HSCs (evaluated by the competitive-repopulating unit (CRU) assay and representing around 3% of the normal pool size) are viable for a prolonged period of time (Pawliuk *et al.*, 1996; U Thorsteinsdottir and GS,

Table 1 Nuclear factors affecting intrinsic HSC properties

Mutated gene	*Hematopoietic phenotype*	*ES cell chimera*	*Hematopoietic chimera*	*Genetic program(s) affected in HSC*		
				Specification	*SR-E*	*SR-M*
Aml1	Embryonic lethality, no effect on primitive hematopoiesis, absence of definitive hematopoiesis (Okuda *et al.*, 1996; Wang *et al.*, 1996a, b, c)	Differentiation into primitive erythrocytes *in vitro*, no contribution to definitive hematopoiesis *in vivo* (Okuda *et al.*, 1996)	Reconstitution (Cai *et al.*, 2000; Ichikawa *et al.*, 2004)	+	?	–
Atf4	Early birth lethality, presence of primitive hematopoiesis, impaired fetal liver hematopoiesis (Masuoka and Townes, 2002)	NA	NA	–	?	?
Bmi-1	Young adulthood lethality, hypoplasia of hematopoietic organs, impaired lymphopoiesis (van der Lugt *et al.*, 1994)	NA	No reconstitution (Lessard and Sauvageau, 2003; Park *et al.*, 2003)	–	–/?	+
Cbfβ	Embryonic lethality, primitive hematopoiesis normal, impaired definitive hematopoiesis (Sasaki *et al.*, 1996; Wang *et al.*, 1996a, b, c)	No contribution to adult hematopoietic tissues (Wang *et al.*, 1996a, b, c)	NA	–	?	?
Cbp	Embryonic lethality, impaired primitive hematopoiesis, impaired early definitive hematopoiesis (Oike *et al.*, 1999; Kung *et al.*, 2000; Tanaka *et al.*, 2000)	Impaired contribution to definitive hematopoiesis (Rebel *et al.*, 2002)	Reduced (Rebel *et al.*, 2002)	–	+/?	–
C/Ebpα	Early birth death, lack mature granulocytes (Zhang *et al.*, 1997)	NA	Reconstitution (Zhang *et al.*, 1997; Lessard and Sauvageau, 2003)	–	–/?	–
C-Myb	Embryonic lethality, primitive hematopoiesis normal, impaired fetal liver hematopoiesis, presence of megakaryocytes (Mucenski *et al.*, 1991; Sumner *et al.*, 2000; Emambokus *et al.*, 2003)	Contribution to early fetal liver hematopoiesis, no contribution to mature hematopoiesis, generation of double-negative thymocytes in Rag-2$^{-/-}$ chimeras (Allen *et al.*, 1999; Clarke *et al.*, 2000; Sumner *et al.*, 2000)	NA	–	–/?	–/?
E2A	High number postnatal death, impaired B-cell development (Bain *et al.*, 1994; Zhuang *et al.*, 1994)	NA	NA	–	?	–
Gata-2	Embryonic lethality, reduced number of primitive erythrocytes, reduced number of yolk sac (YS) colonies (Tsai *et al.*, 1994)	Contribution to primitive erythropoiesis, no contribution to fetal liver, adult hematopoietic organs and mature RBCs, presence of lymphocytes in the spleen (Tsai *et al.*, 1994; Tsai and Orkin, 1997)	NA	–	–/?	–/?
Ikaros	Live up to 4 months, impaired lymphopoiesis, erythropoiesis and myelopoiesis nonaffected (Wang *et al.*, 1996a, b, c; Wu *et al.*, 1997; Lopez *et al.*, 2002; Georgopoulos *et al.*, 1994)	NA	Reduced (Wu *et al.*, 1997; Nichogiannopoulou *et al.*, 1999)	–	–	–
Lmo2	Embryonic lethality, absence of primitive erythropoiesis, presence of YS derived macrophages (Warren *et al.*, 1994)	ES cells are able to form macrophages *in vitro*, no reconstitution to adult hematopoietic tissues (Warren *et al.*, 1994; Yamada *et al.*, 1998)	NA	–	?	?
Meis1	Embryonic lethality, absence of megakaryocytes, reduced fetal liver CFCs (Hisa *et al.*, 2004)	NA	No reconstitution (Hisa *et al.*, 2004)	–	+/?	–/?

Table 1 (*continued*)

Mutated gene	*Hematopoietic phenotype*	*ES cell chimera*	*Hematopoietic chimera*	*Genetic program(s) affected in HSC*		
				Specification	*SR-E*	*SR-M*
Pu.1	Embryonic lethality to early birth death, multilineage defects (Scott *et al.*, 1994; McKercher *et al.*, 1996)	Contribution to fetal erythropoiesis, no contribution to adult hematopoietic tissues (Scott *et al.*, 1997)	No reconstitution (Fisher *et al.*, 1999)	–	?	–
Rae-28	Embryonic lethality, impaired lymphopoiesis (Takihara *et al.*, 1997; Tokimasa *et al.*, 2001)	NA	Reduced (Tokimasa *et al.*, 2001; Ohta *et al.*, 2002)	–	+/?	–
Scl	Embryonic lethality, no development of primitive and definitive hematopoiesis (Robb *et al.*, 1995; Shivdasani *et al.*, 1995)	No contribution to hematopoiesis (Porcher *et al.*, 1996; Robb *et al.*, 1996)	Reconstitution (Mikkola *et al.*, 2003)	+	–	–
T-bet	Lack of Th1 commitment (Finotto *et al.*, 2002)	NA	NA	–	?	–
Tel	Embryonic lethality, similar number of yolk sac (YS) primitive and definitive colonies (Wang *et al.*, 1997)	Contribution to fetal liver hematopoiesis, no contribution to bone marrow myelopoiesis and erythropoiesis, impaired contribution to lymphogenesis, no contribution to adult hematopoietic tissues (Wang *et al.*, 1997, 1998)	NA	–	–	?

BM: bone marrow; CFC: colony-forming cell; ES cell: embryonic stem cell; FL: fetal liver; RBC: red blood cell; SR-E: self-renewal expansion; SR-M: self-renewal maintenance; YS: yolk sac; NA: not applicable. Specific program is affected (+), nonaffected (–), may be affected (+/?) or may not be affected (–/?) by the specific nuclear factor

Figure 2 Symmetrical versus asymmetrical divisions of HSCs. (a) SR-E for self-renewal expansion results from a symmetrical HSC division. (b) SR-M for self-renewal maintenance occurs when HSC undergoes asymmetrical division. This process most likely characterizes adult-type HSCs, which occupy their niche in the bone marrow environment. (c) In this situation, symmetrical division occurs, leading to the production of two differentiated cells (or apoptotic cells) and HSC depletion. This mostly occurs when HSCs are grown *in vitro* under currently available conditions. D: differentiated cell (yellow); S: stem cell (red); SR-E: self-renewal expansion; SR-M: self-renewal maintenance

unpublished observation; see also Sauvageau, Humphries and Iscove, in this issue). In a competitive environment, such as that observed in chimeric mice derived from mutant ES cell implementation, HSCs with impaired SR-E properties may be difficult to detect (Figure 3). Therefore, HSC specification versus SR-E deficiencies could be confused. Figure 3b illustrates an experimental design (involving HSC trans-complementation and *in vivo* reconstitution strategies), which may allow to distinguish between HSC specification and SR-E developmental defects and/or evaluate the 'full' SR-E potential of genetically modified (mutant) HSCs.

Among the genetic factors involved in regulating fetal liver HSC function, the *Meis1* locus, which encodes a homeobox gene of the TALE family, may be specifically involved in regulating SR-E. *Meis1* is highly expressed in fetal liver Sca-1$^+$ Lin$^-$ cells that are enriched for HSC activity (Pineault *et al.*, 2002). *Meis1* null mice are embryonic lethal and display a reduction in fetal liver cell counts and lineage-specific differentiation defects. Furthermore, *Meis1*$^{-/-}$ fetal liver cells are unable to radioprotect lethally irradiated hosts and show an impaired potential to compete in reconstitution assays (Hisa *et al.*, 2004). Further studies including homing and complementation experiments (as those described in Figure 3b) will provide additional information on the role *Meis1* may play in regulating HSC self-renewal.

Overexpression of *Hoxb4* leads to an important *in vivo* and *ex vivo* expansion of HSCs when compared

Experimental condition	Specification defect	SR-E defect	SR-M defect
Loss of Function	Embryonic lethal phenotype. No stem cells are generated.	Mice may be viable. HSCs generated in the embryo sustain the production of differentiated cells.	Mice die as young adults of progressive anemia.
Chimeras	ES cells-derived hematopoiesis is undetectable.	ES cells derived hematopoiesis may be detectable at low levels in embryos and adults. Reduce competitivity in comparison with wild type ES cells.	ES derived HSCs contribute to fetal but poorly to adult hematopoiesis.
Stem Cell Transplantation (adoptive transfer without complementation)	N.A.	No reconstitution unless very large cell doses are administered. Expansion is required for transplantation.	No long-term reconstitution. HSCs are not maintained and therefore no differentiated cells are produced in the transplanted mice.
Adoptive Transfer with Complementation[1]	N.A.	Reconstitution. Complementation and transplantation at limit dilution identify a severe decline in FL HSC numbers (b).	Reconstitution. Complementation and transplantation at limit dilution show normal HSC number in FL (c).

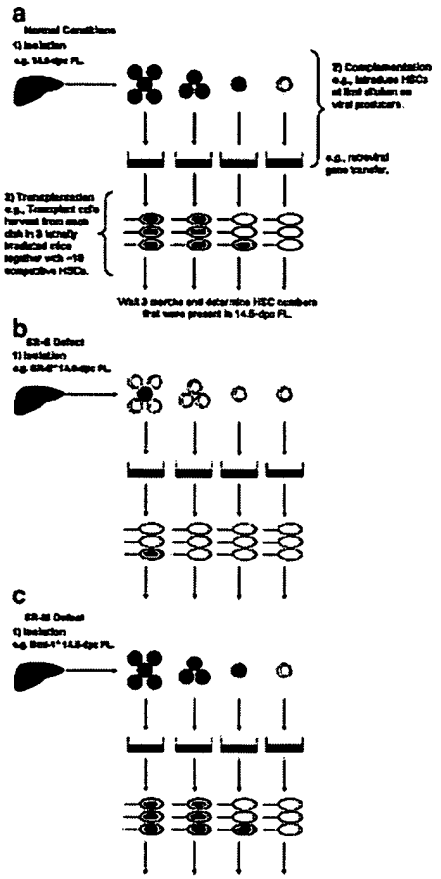

Figure 3 Bases for HSC specification, SR-E and SR-M. (1) Complementation can be performed by reintroducing the gene in 'trans' using, for example, retroviral gene transfer to 14.5-dpc fetal liver cells. Quantitative analysis is performed as indicated in the cartoon. ES cell: embryonic stem cell, FL: fetal liver, SR-E: self-renewal expansion and SR-M: self-renewal maintenance

to nontransduced cells (see Sauvageau, Humphries and Iscove, in this issue). As observed with control HSCs, much of the expansion occurring with *Hoxb4*-transduced cells is observed during the first few weeks following transplantation (Antonchuk *et al.*, 2001). This suggests that the SR-E program, which is operational during embryogenesis, may be reactivated following HSC transplantation and that certain *Hox* genes may play a role in this process (Figure 4). Ongoing studies using *Hox* gene mutant mice should help clarify this issue. Interestingly, HSCs engineered to overexpress an activated form of *STAT3 (STAT3c)* behave similarly to those transduced with *Hoxb4*, potentially suggesting the activation of a common genetic program (Oh *et al.*, 2003). Moreover, Hox proteins interact with Pbx which itself interacts with Meis1 forming a trimeric nuclear complex involved in target gene regulation (Swift *et al.*, 1998; Jacobs *et al.*, 1999; Shen *et al.*, 1999; Liu *et al.*, 2001). The demonstration of a genetic interaction between these transcription factors in the control of fetal liver HSC self-renewal is much awaited.

Thus, much remains to be done to determine whether HSC self-renewal expansion (SR-E) is affected by the loss of function of several transcription factors involved in the specification of early hematopoietic cells (see Table 1). It is hoped that complementary experimental strategies, such as those reported by Mikkola *et al.* (2003) or described in Figure 3b, will help clarify this issue.

Intrinsic regulators of adult HSC self-renewal

Until recently, very little was known about the genetic mechanisms that bring about the intrinsic programs of SR-M in early bone marrow hematopoietic cells. Accumulating evidence, from a number of recent studies, is now pointing to nuclear factors such as the *Polycomb-Group (PcG)* genes *Bmi-1* and *Mph-1/Rae-28*, *GATA-2* and *TEL* for potentially regulating this process. It is likely that these molecular programs will be distinct from those involved in regulating fetal liver HSC SR-E.

Recent studies indicated that the *Polycomb Group (PcG)* gene *Bmi-1* is dispensable for HSC specification and SR-E (both fetal and adult), but absolutely required for their *in vivo* maintenance (SR-M?). During embryonic/fetal liver (FL) (Park *et al.*, 2003) and adult (Lessard *et al.*, 1998; Lessard and Sauvageau, 2003) hematopoiesis, expression of the *Bmi-1* gene is highly enriched in

stem and multipotent progenitor cells (such as primitive human ($CD34^+$ $CD45^-$ $CD71^-$) and mouse ($Sca\text{-}1^+$ Lin^-) bone marrow cells). Nullizygosity for the *Bmi-1* gene in mice leads to severe aplastic anemia due to a progressive impairment of bone marrow HSC function (van der Lugt *et al.*, 1994; Lessard *et al.*, 1999). Consequently, *Bmi-1* null mutants surviving beyond the first week of birth suffer from pneumonia and infections of the intestinal tract and die within 20 weeks of birth. Bone marrow-derived committed progenitors (i.e. myeloid colony-forming cells or CFCs) lacking *Bmi-1* are severely reduced in numbers and in their proliferative potential (van der Lugt *et al.*, 1994). Furthermore, the number and proliferative capacity of primitive myeloid (LTC-IC) and lymphoid (WW-IC) bone marrow progenitors in these mice are severely reduced (to 4 and 1% of wild-type levels, respectively) (Lessard *et al.*, 1999). Retroviral expression of *Bmi-1* in *Bmi-1*$^{-/-}$ fetal liver cells completely rescued the absolute numbers of high and low proliferative potential myeloid colony-forming cells (HPP- and LPP-CFCs) to wild-type levels, indicating that *Bmi-1* is dispensable for the generation of FL-derived myeloid progenitors, but absolutely essential for their full proliferative activity (Lessard and Sauvageau, 2003). Similarly, transplantation studies performed at limiting dilution (LD) in sublethally irradiated recipients confirmed the presence of similar numbers of cells with long-term repopulating potential (LRC) in E14.5 *Bmi-1*$^{-/-}$ fetal livers relative to controls. Importantly, the detection of the *Bmi-1*$^{-/-}$ FL-derived HSCs, at 16 weeks post-transplantation, was strictly dependent on the retroviral expression of *Bmi-1*, indicating that *Bmi-1* is dispensable for the genesis of fetal liver-derived HSCs, but absolutely required for their maintenance. The long-term (16 wks) and pluripotent potential of these cells confirmed that the rescue was occurring at the HSC level (Lessard and Sauvageau, 2003). Clarke and co-workers also demonstrated an inability of bone marrow (BM) and E14.5 FL-derived *Bmi-1*$^{-/-}$ cells to contribute to long-term hematopoiesis in reconstitution (FL and BM) as well as competitive (FL) experiments, suggesting a cell autonomous impairment of their SR-M potential (Park *et al.*, 2003). Of note, the proliferative defect in progenitors derived from *Bmi-1*$^{-/-}$ E14.5 fetal livers (FLs) was much less pronounced than that observed in the bone marrow, suggesting a progressive impairment of the proliferative potential of hematopoietic cells lacking this gene (van der Lugt *et al.*, 1994; Lessard *et al.*, 1999). The apparent progressive instability of the HSC phenotype in the absence of *Bmi-1* may reflect complex epigenetic regulatory circuits established in a context-dependent manner during hematopoiesis. As these results are derived from the analysis of transplantation chimeras, fetal liver and bone marrow, *Bmi-1*$^{-/-}$ HSC SR-M defects are likely cell-autonomous and hematopoietic cell specific. These findings, however, do not exclude additional functions of *Bmi-1* specific to cells of the microenvironment, as these would not be detected in such experimental settings. Together, these studies suggest that *Bmi-1* might be dispensable for HSC specification and SR-E but required for HSC maintenance (Figure 2, SR-M?). Initial experiments indicated that *Bmi-1* lacks the capacity to induce HSC expansion when overexpressed (JL and GS, unpublished observation), reinforcing the notion that *Bmi-1* (SR-M) and *Hoxb4* (SR-E) may regulate distinct self-renewal programs (Figures 2 and 3).

The *Polycomb-Group (PcG)* Mph1/Rae-28 gene product (a known nuclear partner of Bmi-1) also seems to play a key role in sustaining the activity of fetal (and adult?) HSCs (SR-M?). This was demonstrated by a progressive impairment in the numbers and proliferative potential of E14.5 FL-derived CFCs in *Mph1/Rae-28* mutant embryos when compared to wild-type littermates. The absolute number and proliferative potential of primitive myeloid long-term-culture–initiating cells (LTC-ICs) and colony-forming units in spleen ($CFU\text{-}S_{12}$) are also severely decreased (up to 20-fold) in *Mph1/Rae-28*$^{-/-}$ fetal livers when compared to controls. Moreover, serial FL cell transplantation experiments performed at LD in sublethally irradiated congenic mice demonstrated a 15- to 20-fold decrease in CRU content/proliferative activity in *Mph1/Rae-28*$^{-/-}$ embryos relative to controls. These studies indicate a crucial role for *Mph1/Rae-28* in maintaining the activity of HSCs during fetal hematopoiesis (Ohta *et al.*, 2002) and might explain the severe splenic/thymic hypoplasia and perinatal lethality observed in *Mph1/Rae-28*$^{-/-}$ neonates (Takihara *et al.*, 1997; Ohta *et al.*, 2002). The zinc-finger transcription factor *GATA-2*, a member of GATA family that binds a common DNA sequence motif [T/A (GATA) A/G], also plays a critical role in maintaining the pool of multipotential progenitors and HSCs, both during embryogenesis and in the adult (Tsai *et al.*, 1994; Tsai and Orkin, 1997). *GATA-2* gene disruption makes reduction in all hematopoietic precursors, whereas enforced expression of *GATA-2* blocks normal hematopoiesis (Persons *et al.*, 1999). Similarly, the *TEL (translocaction-Ets-leukemia or ETV6)* locus, which encodes an Ets family transcription factor, seems to be required specifically for hematopoiesis of all lineages within the bone marrow (SR-M?) (Wang *et al.*, 1998 and S Orkin, personal communication). Further studies will be necessary to establish whether this defect reflects an inability of *TEL*$^{-/-}$ HSC/progenitors to migrate to the bone marrow or, more likely, to respond appropriately and/or survive within the bone marrow microenvironment.

Seminal work in *Drosophila* revealed that unequal inheritance of specific determinants (such as Numb) is the key to intrinsically determined asymmetric neural progenitor divisions and consequently, neural cell fate specification (see Faubert, Lessard and Sauvageau, in this issue). Whether a similar mechanism underlies HSC SR-M is still unknown. Future work should address whether *Bmi-1*, *Mph1/Rae-28*, *GATA-2* and *TEL* and other yet uncharacterized genetic determinants of HSC SR-M have a role in regulating the asymmetric partitioning of these cell fate determinants during asymmetric HSC divisions.

Bmi-1 is required for the maintenance of leukemic HSCs

There is strong support for the idea that cancer is a stem-cell disease (Dick, 2003). The similarity in the hierarchical organization of malignant and normal tissues is best characterized in the hematopoietic system. Human acute myeloid leukemia (AML) originates from a rare population of primitive cells ($CD34^+$ $CD38^-$) highly enriched in HSCs (Bonnet and Dick, 1997). Most leukemic cells (blasts) are limited in their proliferative capacity and must be constantly replenished by rare, self-renewing 'leukemic stem cells' (L-HSCs). So, like the normal hematopoietic system, leukemia seems to be organized as a hierarchy that originates from a stem-cell pool, which most likely retains remnants of the normal developmental program.

It has also been proposed that the initial, cancer-causing ('transforming') mutations occur in the self-renewing stem cell pool, rather than in already committed precursors. In this view, fewer mutations would be required to generate fully malignant cells if they were to originate from already self-renewing stem cells, as opposed to committed progenitors with low proliferative potential. Thus, two important findings have recently emerged from studies of stem cell biology and carcinogenesis: (1) in the process of neoplastic transformation, the genetic events responsible for disease progression must occur in a stem cell, unless one of the mutations would permit self-renewal in a downstream committed progenitor; (2) within the cancer or leukemia, only a subset of the cells that make up the tumor mass are tumorigenic – the 'cancer stem cells' (reviewed in Reya *et al.*, 2001). These ideas predict similarities in the molecular programs of normal and cancer/AML stem cell self-renewal.

We recently demonstrated that *Bmi-1* is an essential determinant of leukemic stem and progenitor cell maintenance (Lessard and Sauvageau, 2003). Although *Bmi-1* is dispensable for the initial establishment of *Hoxa9-Meis1*-induced AML, replicative exhaustion of *Bmi-1*$^{-/-}$ L-HSCs is reached upon transplantation into secondary recipients, leading to rapid proliferative arrest, differentiation, apoptosis of the leukemic blasts and resumption to normal host hematopoiesis. However, through high *in vitro* selective pressure, which induced epigenetic alterations of critical regulators of proliferation (including both $p19^{ARF}$ and $p16^{INK4a}$), rare immortalized *Bmi-1*$^{-/-}$ highly proliferative clones (HPCs) could be derived from *Bmi-1*$^{-/-}$ AMLs (at a frequency of 1 versus 24% without selection in control AMLs). When assessed *in vivo*, some of these *Bmi-1*$^{-/-}$ HPCs, initially nonleukemogenic, eventually acquired the capacity to induce AML, pointing to clonal evolution rather than selection, as the underlying mechanism responsible for leukemic progression in certain *Bmi-1*$^{-/-}$ HPCs. Retroviral introduction of *Bmi-1* in these 'immortal' but nonleukemic *Bmi-1*$^{-/-}$ HPCs could induce AML within similar latency periods than HPCs derived from control AMLs. The highly polyclonal nature and short latencies of these leukemias indicated that *Bmi-1* is sufficient to fully restore the leukemogenic potential of these clones. This demonstrates that *Bmi-1*'s function in L-HSCs involves more than cellular proliferation, which was apparently similar

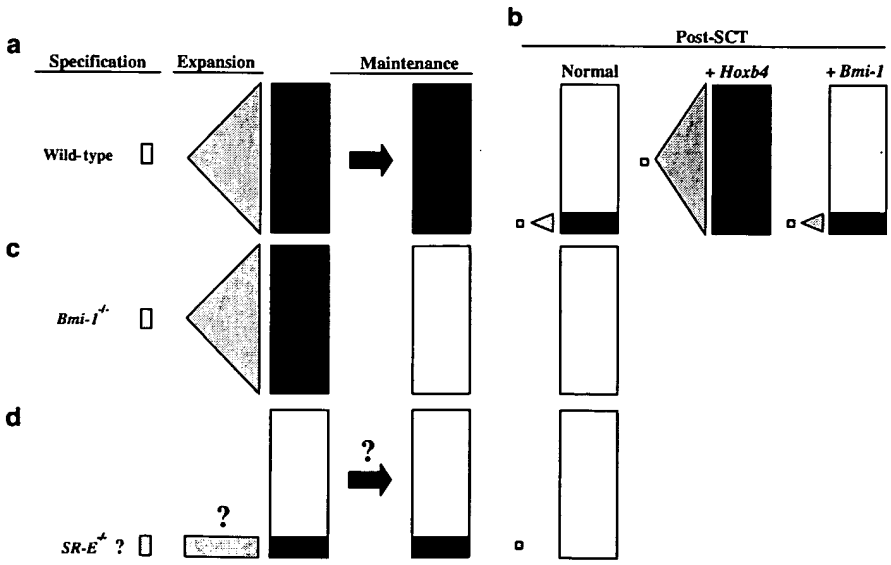

Figure 4 Self-renewal programs regulated by Bmi-1 and Hoxb4. (a) Wild-type HSCs are generated (specification: yellow), expand in the embryo (expansion: orange) and are maintained during adult life (maintenance: red). (b) Wild-type stem cells can be transplanted into myeloablated recipient, where they reconstitute a fraction of the stem cell pool (post-SCT normal). HSCs overexpressing *Hoxb4* can reconstitute 100% of the stem cell pool after bone marrow transplantation into myeloablated recipient (post-SCT + *Hoxb4*) and overexpression of *Bmi-1* has no effect on the stem cell pool (post-SCT + *Bmi-1*). (c) HSCs lacking *Bmi-1* are generated, expanded but are not maintained through adult life. Since HSCs are not maintained, no stem cells transplantation is possible. (d) Stem cells deficient in the genetic program SR-E are generated but may fail to expand. As a result, HSCs cannot be transplanted into myeloablated recipient. SCT: stem cell transplantation

between the several selected HPCs derived from control and *Bmi-1*$^{-/-}$ AMLs (Lessard and Sauvageau, 2003).

Importantly, epigenetic and genetic abrogation of *CDKN2A* (*p16*^{INK4a}) and *CDKN2B* (*p19*^{ARF}) are common lesions associated with poor prognosis in several human leukemias and mouse leukemia models (Hebert *et al.*, 1994; Maloney *et al.*, 1999; Schmitt *et al.*, 1999; Carter *et al.*, 2001; Garcia *et al.*, 2002). The observation that the leukemogenic potential of *Bmi-1*$^{-/-}$ clones correlated with the loss of expression of *p16*^{INK4a} and *p19*^{ARF} underscores the importance of investigating whether these CKIs represent downstream regulators of *Bmi-1* function in normal and neoplastic HSCs. However, the inability of several *Bmi-1*$^{-/-}$ clones, which lacked expression of both *p16*^{INK4a} and *p19*^{ARF} (and other CKIs), to induce AML when transplanted indicates that Bmi-1's function in leukemic stem cells involves additional targets (Lessard and Sauvageau, 2003). Together, these studies indicate that *Bmi-1* is dispensable for the generation of normal and leukemic fetal liver-derived HSCs, but is absolutely necessary for their SR-M.

Bmi-1: a common regulator of stem cell function?

The genetic mechanisms regulating self-renewal of the HSCs may be more generally applicable to other regenerating tissue systems. Recent findings implicated the *Notch* (Austin and Kimble, 1987; Henrique *et al.*, 1997; Varnum-Finney *et al.*, 2000), *Wnt* and *Shh* (Bhardwaj *et al.*, 2001; Wechsler-Reya and Scott, 2001; Zhang and Kalderon, 2001) signaling pathways in promoting stem cell self-renewal in a variety of different epithelia in addition to HSCs. Interestingly, mutations of these pathways have been associated with a number of human neoplasia, including colon carcinoma and epidermal tumors (Chan *et al.*, 1999; Polakis, 2000) *(Wnt)*, medulloblastoma and basal call carcinoma (Gailani and Bale, 1999; Wechsler-Reya and Scott, 2001) *(Shh)*, and T-cell leukemias (Ellisen *et al.*, 1991) *(Notch)*. Supporting a potential role for *Bmi-1* in regulating the SR-M of stem cells others than the HSCs, severe skeletal and neuronal developmental defects were reported in the *Bmi-1* knockout mouse (van der Lugt *et al.*, 1994). Moreover, the overexpression of *Bmi-1* was observed in several cases of human non-small-cell lung cancer (Vonlanthen *et al.*, 2001), breast cancer cell lines (Dimri *et al.*, 2002) as well as immortalized mammary epithelial cells (MECs) (Dimri *et al.*, 2002). High-level DNA amplifications and gains of the region encompassing the human *Bmi-1* gene locus (10p13) have also been reported in several cases of head and neck carcinomas (Knuutila *et al.*, 1998). Future experiments will reveal whether the function of *Bmi-1* in regulating HSC SR-M extends to stem cells in other self-renewing tissues.

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